

# Study Plan of Aquatic Toxicity Test

## Appendix 3.3

**Marine Eco-Technology Institute Co., Ltd.**

# **1. AQUATIC TOXICITY TEST PROCEDURE**

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## 1. AQUATIC TOXICITY TEST PROCEDURE

The whole test procedure for Aquatic toxicity test is shown on follow flow chart in Fig. 1. The sample of control (non-treated seawater) and de-ballast water (treated seawater) from BioViolet™ is collected by 600 L (control water) and 400 L (de-ballast water), respectively. The samples are transferred to MEI Laboratory using refrigerator car. The treated seawater is diluted on the control seawater (non-treated ballast water of the BioViolet™ as crude liquid for making the test concentration (dilution series). The six test concentrations including control and 100.00 % de-ballast water are arranged for the final definitive experiment. More detail test procedure on each step is shown in Study Plans and/or SOPs for aquatic toxicity test.

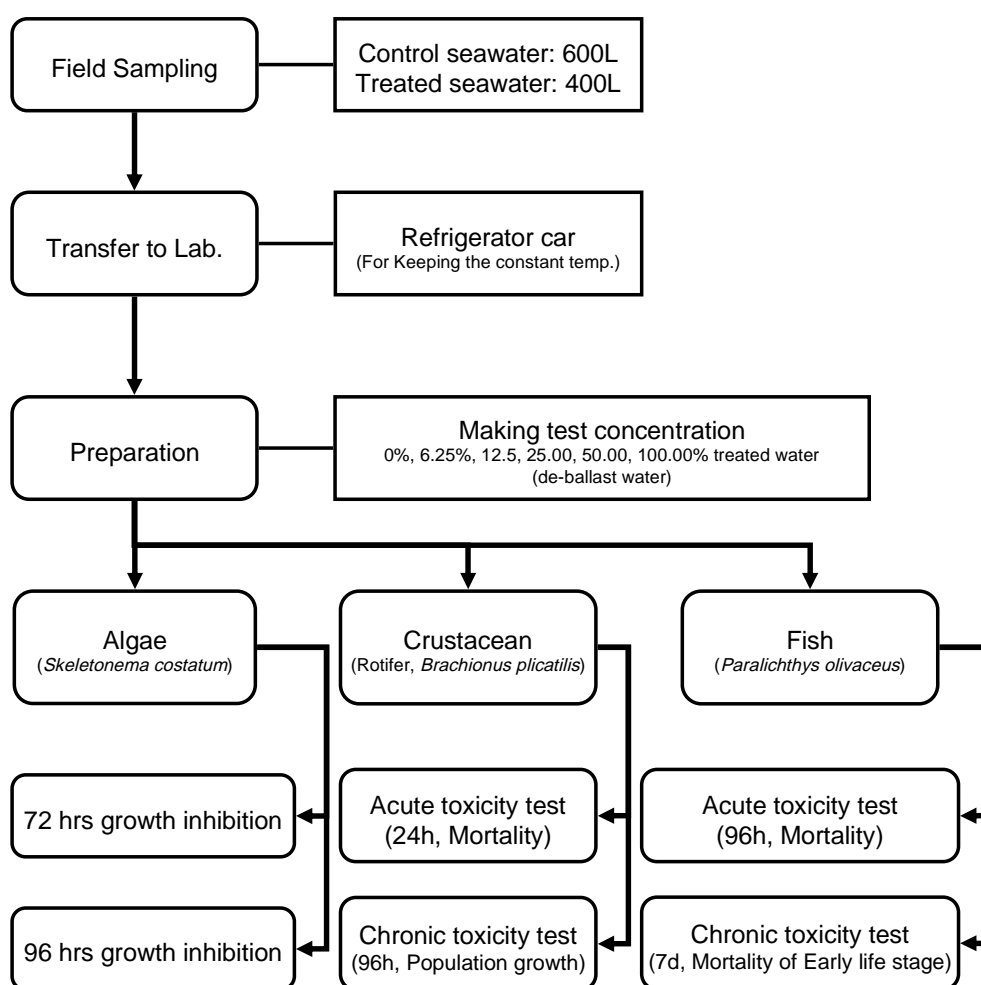


Fig. 1. Flow chart of the aquatic toxicity test procedure.

## **2. STUDY PLAN**

### **2.1 Algae Growth Inhibition Test**

#### **2.1.1 ANNEX 1**

***Skeletonema costatum*, Growth Inhibition Test - 72 hours**



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# STUDY PLAN

**Aquatic Toxicity For > 32 & 3-32 psu Treated Ballast Water of  
BioViolet™**

**- *Skeletonema costatum*, Growth Inhibition Test - 72 hours**

**Study Name: BioViolet™**

**Study No: BW- DBWT1107-KS**

Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## STUDY PLAN PREPARATION AND APPROVAL

Study title: Algae growth inhibition test-72h to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioViolet™ toward *Skeletonema costatum*

Study name: BioViolet™ Study No: BW-DBWT1107-KS

Test facility Marine Eco-technology Institute Co., Ltd.

2011. 02. 28.

Study Director: Myung-Baek Shon

(signature)

Lab. Manager: Myung-Baek Shon

(signature)

Project Officer: Min Ho Son

(signature)

Organization Korean Marine Equipment Research Institute

2011. 02. 28.

Test Facility Manager: Jun-Hak Lee

(signature)

Project Director: Young-Soo Kim

(signature)

Kwang San Co., Ltd.

2011. 02. 28.

Monitor: Sang Bum Bae

(signature)

Sponsor: Young Woo Lee

(signature)

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# **1. OUTLINE OF STUDY**

## **1.1 Purpose**

The purpose of this study is to assess the toxic effects of the  $> 32$  and  $3-32$  psu ballast water treated by the BioViolet™ (de-ballast water from the BWMS) on the growth of diatom, *Skeletonema costatum*.

## **1.2 Principle**

Mono-specific *Skeletonema costatum* strains are cultured for several generations in a defined medium containing a range of concentrations of treated ballast water, prepared by mixing appropriate quantities of nutrient concentrate, seawater, concentration series of the treated ballast water (test solution), and an inoculum of exponentially growing *S. costatum* cells. The test solutions are incubated for a period of  $72 \text{ h} \pm 2 \text{ h}$ , during which the cell density in each is measured at intervals of at least every  $24 \text{ h} \pm 2 \text{ h}$ . Inhibition is measured as a reduction in specific growth rate, relative to control cultures grown under identical conditions.

## **1.3 Good Laboratory Practice**

All procedure of this study is complied with following GLP regulation:

- “OECD Principles of Good Laboratory Practice”

Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

## **1.4 Guideline**

This study is conducted on the basis of principle of following standard method and applied for *Skeletonema costatum*:

- “ISO 10253 – Water quality – marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*”

INTERNATIONAL STANDARD, (Adapted: 2006-04-15, Second edition)

## **1.5 Sponsor**

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

Tel: +82-51-974-6351

Fax: +82-51-974-6405

## **1.6 Test facility**

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

Tel: + 82-51-611-6200

Fax: + 82-51-611-0588

## 1.7 Test site

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## 1.8 Study director

Name: Myung-Baek Shon

Department: BAT (Bio-assay Team for aquatic organisms)

## 1.9 Study timetable

### 1.9.1 Treated ballast water (3-32psu) and non-Treated ballast water (3-32psu)

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Reference toxicant test	2011. 08. 08 ~ 08. 11
Acclimation	2011. 02. 28 ~ 09. 04
Pre-culture for test	2011. 09. 04
Collect of sample	2011. 09. 06
Experimental start	2011. 09. 07
Exposure date	2011. 09. 07
Evaluation of cell density	2011. 09. 07 ~ 09. 10
Evaluation of chlorophyll <i>a</i>	2011. 09. 08 ~ 09. 11
Experimental completion	2011. 09. 10
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### 1.9.2 Treated ballast water (> 32 psu) and non-Treated ballast water (> 32 psu)

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Acclimation	2011. 02. 28 ~ 07. 10
Pre-culture for test	2011. 07. 10
Collect of sample	2011. 10. 31
Experimental start	2011. 11. 01
Exposure date	2011. 11. 01

Evaluation of cell density	2011. 11. 01 ~ 11. 04
Evaluation of chlorophyll <i>a</i>	2011. 11. 02 ~ 11. 05
Experimental completion	2011. 11. 04
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### **1.10 Responsible personnel**

Laboratory manager (Test site manager)	Myung-Baek Shon
Statistical analysis	
Culture of test organisms	Tae Won Kim
Observation and record	
Sampling, transit and custody of test substance or solution	
Disposal of the resting substance	Je Kwan Park
Check the chemical condition	
Disposal of the resting organisms	Hyeong Ju Seok
Sampling, transit and custody of test substance or solution	

### **1.11 Retention of records and data**

#### **1.11.1 Duration**

Duration of storage is 5 years after end of the study. It will be decided that storage of data after 5 years should be consulted with the client.

#### **1.11.2 Place of storage**

Name: Archives of Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

#### **1.11.3 Retention of records and data**

Study plan, final report, raw data, documents related with the study, communication documents, and etc.

## 2. MATERIALS AND METHODS

### 2.1 Test substance-1 (Treated ballast water (3-32psu) -Treated)

2.1.1 Substance name

Treated water

2.1.2 Lot No.

none

2.1.3 Type

liquid

2.1.4 Principal ingredients

Seawater

2.1.5 Temperature

14~27 °C

2.1.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.1.8 Custody condition

Constant room at 4 °C for 7 days

2.1.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.1.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.1.11 Disposal of the resting substances

Scrap after test

2.1.12 Certificate of analysis

Client supplies to test organization

## **2.2 Test substance-2 (Non-treated ballast water (3-32psu) -Control)**

2.2.1 Substance name

Non-treated water

2.2.2 Lot No.

none

2.2.3 Type

liquid

2.2.4 Principal ingredients

Seawater

2.2.5 Temperature

14~27 °C

2.2.6 Salinity

3-32 psu

2.2.7 pH

6~8

2.2.8 Custody condition

Constant room at 4 °C for 7 days

2.2.9 Supplier

Name: Kwang San Co., Ltd

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.2.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.2.11 Disposal of the resting substances

Scrap after test

2.2.12 Certificate of analysis

Client supplies to test organization

## **2.3 Test substance-3 (Treated ballast water (> 32 psu) -Treated)**

2.3.1 Substance name

Treated water

2.3.2 Lot No.

none

2.3.3 Type

liquid

2.3.4 Principal ingredients

Seawater

2.3.5 Temperature

14~27 °C

2.3.6 Salinity

>32 psu

2.3.7 pH

6~8

2.3.8 Custody condition

Constant room at 4 °C for 7 days

2.3.9 Supplier

Name: Kwang San Co., Ltd

Address: 452-7 Nae-dong, Seongsan-gu, Changwon, Gyeongsangnam-do, Korea 641-050

2.3.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.3.11 Disposal of the resting substances

Scrap after test

2.3.12 Certificate of analysis

Client supplies to test organization

## **2.4 Test substance-4 (Non-treated ballast water (> 32 psu) -Control)**

2.4.1 Substance name

Non-treated water

2.4.2 Lot No.

none

2.4.3 Type

liquid

2.4.4 Principal ingredients

Seawater

2.4.5 Temperature

14~27 °C

2.4.6 Salinity

>32 psu

2.3.7 pH

6~8

2.4.8 Custody condition

Constant room at 4 °C for 7 days

2.4.9 Supplier

Name: Kwang San Co., Ltd

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.4.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.4.11 Disposal of the resting substances

Scrap after test

2.4.12 Certificate of analysis

Client supplies to test organization

## 2.5 Preparation of the test substance (SOP#TP-011)

The concentration of test substance is diluted on the control seawater (non-treated ballast water) as crude liquid. The six concentrations of test substance including control (0.00 %, only non-treated ballast water) and 100.00 % are arranged for the final definitive experiment. pH is not adjusted by  $8.0 \pm 0.2$  because we have assumed that acidification and/or alkalization of test water treated by BioViolet™ is also toxic effect.

## 2.6 Culture condition (SOP#TO-001)

### 2.6.1 Room

Incubator

### 2.6.2 Vessel type / volume

Glass flask / 250 mL

### 2.6.3 Medium volume / vessel volume & medium

100 mL/250 mL, f/2 (see Annex 2)

### 2.6.4 Inoculum density

$5 \times 10^3$  cells/mL

### 2.6.5 Temperature

20 °C

### 2.6.6 Interval of inoculum

Every a week

### 2.6.7 L:D cycle

Continuous light

### 2.6.8 Intensity of light

3,000 lux

## 2.7 Test system

### 2.7.1 Test organisms

#### .1 Scientific name

*Skeletonema costatum* (VBA-007)

#### .2 Producer

Marine Eco-technology Institute Co., Ltd., Republic of Korea

#### .3 Dealer

Marine Eco-technology Institute Co., Ltd., Republic of Korea

### 2.7.2 Ground of selected test organisms

*Skeletonema costatum* is important as primary producer in trophic structure and widely distributed phytoplankton species (Phylum Bacillariophyta) in estuarine and coastal areas. It is used to evaluate the aquatic toxicity and has a large database.



### 2.7.3 Isolation of phytoplankton (SOP#TO-001)

Isolation method	Dilution techniques
Species origin	Yongho Bay in Korea

### 2.7.4 Preparation of pre-culture and inoculation (SOP#TP-008)

A pre-culture is started 2 to 4 days before the beginning of the experiment. In order to maintain the exponential growth phase until the start of the experiment, a pre-culture is made to inoculate  $1 \times 10^4$  cells/mL from the algal stock culture to the growth medium. The pre-culture is incubated under the same conditions as those in the experiment. And the pre-culture for the experiment with 3-32 psu treated ballast water is carried out same way after acclimation of test organisms for more than 14 days.

### 2.7.5 Check the test organisms for the experiment (SOP#TP-001)

The cell density in the pre-culture is immediately measured before use, in order to calculate the required inoculation volume and appearance of cells.

### 2.7.6 Disposal of the resting test organisms (SOP#TO-006)

The resting test organisms are scrapped after sterilization by the autoclave.

## 2.8 Study design

### 2.8.1 Test concentration (SOP#TP-011)

Control	Control seawater	100.00 %: Seawater treated by BioViolet™	0.00 %
6.25 %	Control seawater	93.75 %: Seawater treated by BioViolet™	6.25 %
12.50 %	Control seawater	87.50 %: Seawater treated by BioViolet™	12.50 %
25.00 %	Control seawater	75.00 %: Seawater treated by BioViolet™	25.00 %
50.00 %	Control seawater	50.00 %: Seawater treated by BioViolet™	50.00 %
100.00 %	Control seawater	0.00 %: Seawater treated by BioViolet™	100.00 %

### 2.8.2 Exposure conditions (SOP#TP-001)

The final cell density ( $3 \times 10^3$  cells/mL) is added from the algal pre-culture to the each concentration in the test vessels. And then the test vessels are incubated for 72 hours at  $22 \pm 1^\circ\text{C}$ .

Experiment conditions and criteria are shown Annex 3 and application site is shown Annex 5.

### 2.8.3 Observation item and measurement components

#### .1 Biomass (SOP#TP-009, SOP#TP-012)

Measurement of biomass is conducted by measuring chlorophyll *a* extracted with 90% acetone using Tuner Designs AU-10. The measurement of the chlorophyll *a* is immediately done within 2 hours after

extraction of it. To obtain the regression equation between chlorophyll *a* and cell density, cell count in the control is done by manual cell counting used the microscope (CKX 31, Olympus) with counting chamber.

Calculations of the growth and the inhibition growth rate are shown Annex 4.

.2 Other observations (SOP#TP-001)

Microscopic observation is performed to verify normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae at the end of the experiment.

.3 pH (SOP#TE-002)

pH of the solutions at the beginning and at the end of the experiment is measured.

## 2.9 Reference toxicant test

.1 Test substance: potassium dichromate (CAS No.: 7778509)

.2 The test procedure and condition

The test procedure and condition of reference toxicant test is same to '2.7 Test system' '2.8.2 Exposure conditions' and '2.8.3 Observation item and measurement component' in this study.

.3 Concentrations: Control, 0.625, 1.25, 2.50, 5.00, 10.00 mg/L

.4 Precision objects (see Annex 3)

End point: 72hour growth inhibition (72h-EC50)

Mean (Standard deviation) value: 2.5 ( $\pm 1.1$ ) mg/L

## 2.10 Statistical procedures (SOP#SP-101, SOP#SP-102)

For satisfactory correlation with biomass, chlorophyll *a* and cell density in the control are analyzed by linear correlation analysis with Excel program and the greatest  $R^2$  value is yielded. Cell density is calculated by equation from the linear correlation analysis.

NOEC, LOEC and EC50 values of the end point are estimated by statistical analysis procedure referred from USEPA (2002). TOXCALC 5.0 program (Tidepool scientific software, USA) is used for statistic analysis.

## 3. REFERENCES

ISO 10253. 2006. International Standard, water quality – marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*. 12pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

## ANNEX 1

### Definitions

- 1. Coefficient of variation** is a dimensionless measure of the variability of a parameter, defined as the ratio of the standard deviation to the mean. This can also be expressed as a percent value. Mean coefficient of variation of average specific growth rate in replicate control cultures should be calculated as follows:

  1. Calculate % CV of average specific growth rate out of the daily/section by section growth rates for the respective replicate;
  2. Calculate the mean value out of all values calculated under point 1 to get the mean coefficient of variation of the daily/section by section specific growth rate in replicate control cultures.
- 2. EC<sub>x</sub>** is the concentration of the test substance dissolved in test medium that results in an x % (e.g. 50 %) reduction in growth of the test organisms within a stated exposure period.
- 3. Growth rate** (average specific growth rate) is the logarithmic increase in biomass during the exposure period.
- 4. Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant reducing effect on growth (at  $p < 0.05$ ) when compared with the control, within a given exposure time.
- 5. No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC.
- 6. Specific growth rate** is a response variable defined as quotient of the difference of the natural logarithms of a parameter of observation and the respective time period.
- 7. Pre-culture** is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of 2 to 4 days.
- 8. Biomass** is the dry weight of living matter present in a population expressed in terms of a given volume; e.g., mg algae/litre test solution. Usually “biomass” is defined as a mass, but in this study this word is used to refer to mass per volume. Also in this study, surrogates for biomass as cell density (cells/mL) from fluorescence method.

## ANNEX 2

Composition of f/2 (Guillard & Ryther 1962, Guillard 1975)

Component	Stock solution	Quantity Used	Concentration in Final Medium (M)
NaNO <sub>3</sub>	75.0 g/L dH <sub>2</sub> O	1.0 mL	$8.82 \times 10^{-4}$
NaH <sub>2</sub> PO <sub>4</sub> ·4H <sub>2</sub> O	5.0 g/L dH <sub>2</sub> O	1.0 mL	$3.62 \times 10^{-5}$
Na <sub>2</sub> SiO <sub>3</sub> ·9H <sub>2</sub> O	30.0 g/L dH <sub>2</sub> O	2.0 mL	$1.06 \times 10^{-4}$
* f/2 Trace metal solution	(see recipe below)	1.0 mL	-
** f/2 Vitamin solution	(see recipe below)	0.5 mL	-
* f/2 Trace metal solution			
Component	Stock solution	Quantity Used	Concentration in Final Medium (M)
FeCl <sub>3</sub> ·6H <sub>2</sub> O	-	3.15 g	$1.17 \times 10^{-5}$
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	-	4.36 g	$1.17 \times 10^{-5}$
CuSO <sub>4</sub> ·5H <sub>2</sub> O	9.8 g/L dH <sub>2</sub> O	1.0 mL	$3.93 \times 10^{-8}$
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	6.3 g/L dH <sub>2</sub> O	1.0 mL	$2.60 \times 10^{-8}$
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	22.0 g/L dH <sub>2</sub> O	1.0 mL	$7.65 \times 10^{-8}$
CoCl <sub>2</sub> ·6H <sub>2</sub> O	10.0 g/L dH <sub>2</sub> O	1.0 mL	$4.20 \times 10^{-8}$
MnCl <sub>2</sub> ·4H <sub>2</sub> O	180.0 g/L dH <sub>2</sub> O	1.0 mL	$9.10 \times 10^{-7}$
** f/2 Vitamin solution			
Component	Stock solution	Quantity Used	Concentration in Final Medium (M)
Cyanocobalamin (Vitamin B <sub>12</sub> )	1.0 g/L dH <sub>2</sub> O	1.0 mL	$3.69 \times 10^{-10}$
Biotin (Vitamin H)	0.1 g/L dH <sub>2</sub> O	10.0 mL	$2.05 \times 10^{-9}$
Thiamine HCl (vitamin B <sub>1</sub> )	-	200.0 mL	$2.96 \times 10^{-7}$

### ANNEX 3

Experiment conditions and acceptability criteria for diatom, *Skeletonema costatum*, acute growth inhibition tests with the > 32 and 3-32 psu de-ballast water from the BioViolet™

Test parameter	Conditions
Test type	Static
Temperature	22 ± 1 °C
Light quality	“Cool white” fluorescent lamp
Light intensity	3,000 lux
Photoperiod	Continuous illumination
Test chamber size	15 mL
Test solution volume	10 mL
Renewal of test solutions	None
Initial cell density in test chambers	3,000 cells/mL
No. replicate chambers per concentration	3
Shaking rate	Twice daily by hand
Aeration	None
Dilution water	Filtered seawater (Control seawater) by 0.2µm membrane filter
Test concentrations	Effluents: 6 including receiving water 100.00% and a control
Test dilution factor	Effluents: 0.5
Test duration	72 hours
Endpoint	Growth inhibition
Test acceptability criteria	Specific growth rate in the control: $\geq 0.92 \text{ day}^{-1}$ Variation coefficient of the control specific growth rate during the whole test period: $\leq 7\%$
Precision object	Reference toxicant: potassium dichromate 72h EC50 value: 1.4~3.6 mg/L

## ANNEX 4

**1. The average specific growth rate for a specific period is calculated from equation [1]:**

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (\text{day}^{-1}) \quad [1]$$

where:

$\mu_{i-j}$  is the average specific growth rate from time I to j;

$X_i$  is the biomass at time  $i$ ;

$X_j$  is the biomass at time  $j$ ;

**2. The percent inhibition of growth rate for each treatment replicate is calculated from equation [2]:**

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad [2]$$

where:

$\%I_r$  is percent inhibition in average specific growth rate;

$\mu_C$  is mean value for average specific growth rate ( $\mu$ ) in the control group ;

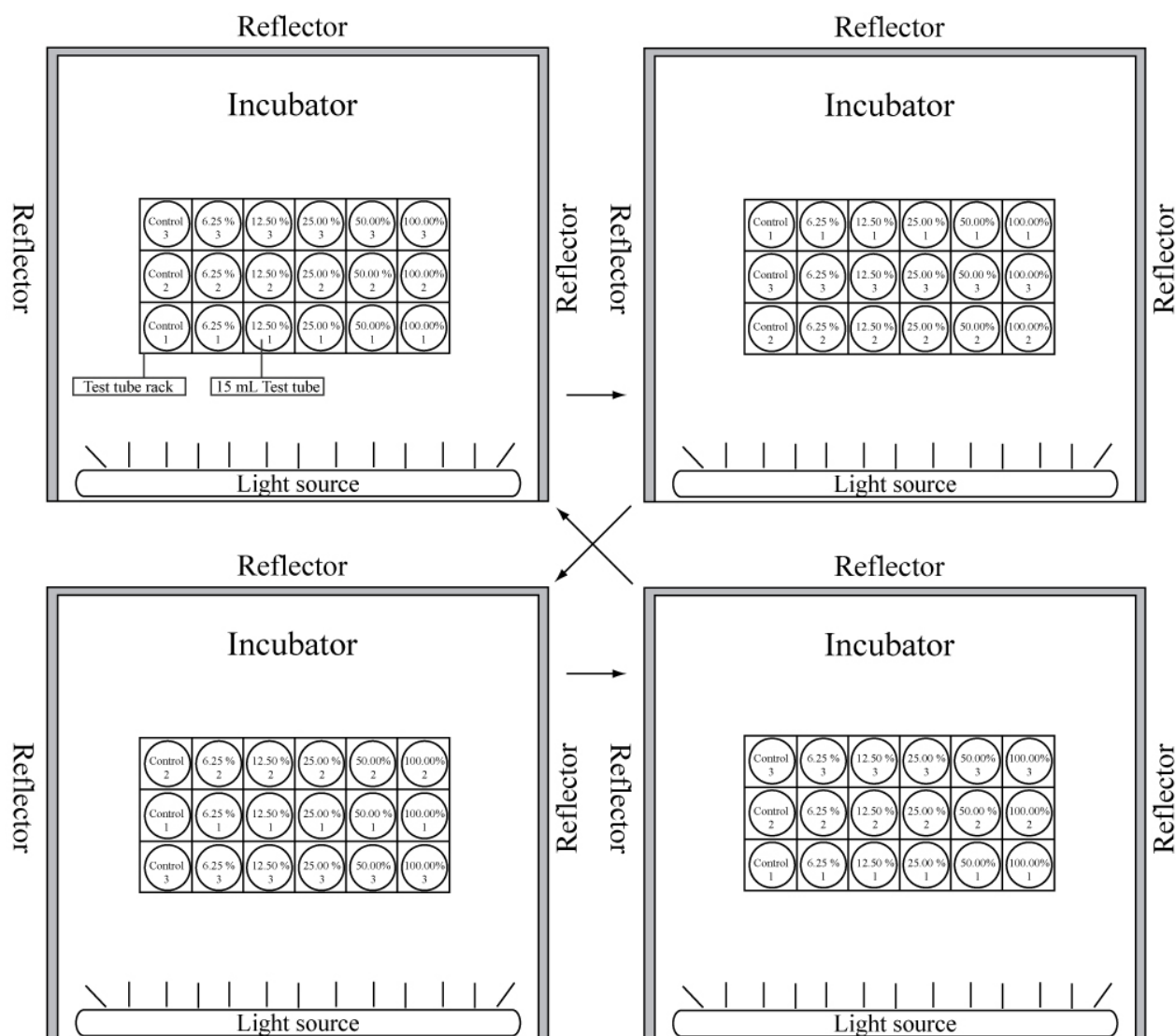
$\mu_T$  is average specific growth rate ( $\mu$ ) in treatment replicate

Each treatment group and control group are calculated a mean value for growth rate along with variance estimates.

## ANNEX 5

### Application Site

Application site for the acute toxicity test with diatom, *Skeletonema costatum* is shown as follow. The arrowheads indicate the systematical rotation of the test vessels.



## **2.1.2 ANNEX 2**

***Skeletonema costatum*, Growth Inhibition Test - 96 hours**





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# STUDY PLAN

**Aquatic Toxicity For > 32 & 3-32 psu Treated Ballast Water of  
BioViolet™**

**- *Skeletonema costatum*, Growth Inhibition Test - 96 hours**

**Study Name: BioViolet™**

**Study No: BW- DBWT1107-KS**

Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## STUDY PLAN PREPARATION AND APPROVAL

Study title: Algae growth inhibition test-96h to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioViolet™ toward *Skeletonema costatum*

Study name: BioViolet™ Study No: BW-DBWT1107-KS

Test facility Marine Eco-technology Institute Co., Ltd.

2011. 02. 28.

Study Director: Myung-Baek Shon

(signature)

Lab. Manager: Myung-Baek Shon

(signature)

Project Officer: Min Ho Son

(signature)

Organization Korean Marine Equipment Research Institute

2011. 02. 28.

Test Facility Manager: Jun-Hak Lee

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Project Director: Young-Soo Kim

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Kwang San Co., Ltd.

2011. 02. 28.

Monitor: Sang Bum Bae

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Sponsor: Young Woo Lee

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# 1. OUTLINE OF STUDY

## 1.1 Purpose

The purpose of this study is to assess the toxic effects of the  $> 32$  and  $3-32$  psu ballast water treated by BioViolet™ (de-ballast water from the BWMS) on the growth of diatom, *Skeletonema costatum*.

## 1.2 Principle

Mono-specific *Skeletonema costatum* strains are cultured for several generations in a defined medium containing a range of concentrations of treated ballast water, prepared by mixing appropriate quantities of nutrient concentrate, seawater, concentration series of the treated ballast water (test solution), and an inoculums of exponentially growing *S. costatum* cells. The test solutions are incubated for a period of  $96 \text{ h} \pm 2 \text{ h}$ , during which the cell density in each is measured at intervals of at least every  $24 \text{ h} \pm 2 \text{ h}$ . Recommended test period in the ISO10253 is  $72 \text{ h} \pm 2 \text{ h}$ . However, 24 h of test period in this study is extended to clarify the toxic effect when *S. costatum* is exposed to treated ballast water for a longer than 72 h. Inhibition is measured as a reduction in specific growth rate, relative to control cultures grown under identical conditions.

## 1.3 Good Laboratory Practice

All procedure of this study is complied with following GLP regulation:

- “OECD Principles of Good Laboratory Practice”

Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

## 1.4 Guideline

This study is conducted on the basis of principle of following standard method and applied for *Skeletonema costatum*:

- “ISO 10253 – Water quality – marine algal growth inhibition test with *Skeletonema costatum* and *Phaeodactylum tricornutum*”

INTERNATIONAL STANDARD, (Adapted: 2006-04-15, Second edition)

## 1.5 Sponsor

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

Tel: +82-51-974-6351

Fax: +82-51-974-6405

## 1.6 Test facility

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

Tel: + 82-51-611-6200

Fax: + 82-51-611-0588

## 1.7 Test site

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## 1.8 Study director

Name: Myung-Baek Shon

Department: BAT (Bio-assay Team for aquatic organisms)

## 1.9 Study timetable

### 1.9.1 Treated ballast water (3-32 psu) and non-Treated ballast water (3-32 psu)

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Acclimation	2011. 02. 28 ~ 09.04
Pre-culture for test	2011. 09. 04
Collect of sample	2011. 09. 06
Experimental start	2011. 09. 07
Exposure date	2011. 09. 07
Evaluation of cell density	2011. 09. 07 ~ 09. 11
Evaluation of chlorophyll <i>a</i>	2011. 09. 08 ~ 09. 12
Experimental completion	2011. 09. 11
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### 1.9.2 Treated ballast water (> 32 psu) and non-Treated ballast water (> 32 psu)

Study initiation	2011. 02. 28
Batch culture of test organisms	2011. 02. 28
Acclimation	2011. 02. 28 ~ 07. 16
Pre-culture for test	2011. 10. 29
Collect of sample	2011. 10. 31
Experimental start	2011. 11. 01
Exposure date	2011. 11. 01
Evaluation of cell density	2011. 11. 01 ~ 11. 05

Evaluation of chlorophyll <i>a</i>	2011. 11. 02 ~ 11. 06
Experimental completion	2011. 11. 05
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### **1.10 Responsible personnel**

Laboratory manager (Test site manager)	Myung-Baek Shon
Statistical analysis	
Culture of test organisms	Tae Won Kim
Observation and record	
Sampling, transit and custody of test substance or solution	
Disposal of the resting substance	Je Kwan Park
Check the chemical condition	
Disposal of the resting organisms	Hyeong Ju Seok
Sampling, transit and custody of test substance or solution	

### **1.11 Retention of records and data**

#### **1.11.1 Duration**

Duration of storage is 5 years after end of the study. It will be decided that storage of data after 5 years should be consulted with the client.

#### **1.11.2 Place of storage**

Name: Archives of Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

#### **1.11.3 Retention of records and data**

Study plan, final report, raw data, documents related with the study, communication documents, and etc.

## **2. MATERIALS AND METHODS**

### **2.1 Test substance-1 (Treated ballast water (3-32 psu) -Treated)**

2.1.1 Substance name

Treated water

2.1.2 Lot No.

none

2.1.3 Type

liquid

2.1.4 Principal ingredients

Seawater

2.1.5 Temperature

14~27 °C

2.1.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.1.8 Custody condition

Constant room at 4 °C for 7 days

2.1.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.1.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.1.11 Disposal of the resting substances

Scrap after test

2.1.12 Certificate of analysis

Client supplies to test organization

## **2.2 Test substance-2 (Non-treated ballast water (3-32 psu) -Control)**

2.2.1 Substance name

Non-treated water

2.2.2 Lot No.

none

2.2.3 Type

liquid

2.2.4 Principal ingredients

Seawater

2.2.5 Temperature

14~27 °C

2.2.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.2.8 Custody condition

Constant room at 4 °C for 7 days

2.2.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.2.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.2.11 Disposal of the resting substances

Scrap after test

2.2.12 Certificate of analysis

Client supplies to test organization



### **2.3 Test substance-3 (Treated ballast water (> 32 psu) -Treated)**

2.3.1 Substance name

Treated water

2.3.2 Lot No.

none

2.3.3 Type

liquid

2.3.4 Principal ingredients

Seawater

2.3.5 Temperature

14~27 °C

2.3.6 Salinity

3-32 psu

2.3.7 pH

6~8

2.3.8 Custody condition

Constant room at 4 °C for 7 days

2.3.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.3.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.3.11 Disposal of the resting substances

Scrap after test

2.3.12 Certificate of analysis

Client supplies to test organization

## **2.4 Test substance-4 (Non-treated ballast water (> 32 psu) -Control)**

2.4.1 Substance name

Non-treated water

2.4.2 Lot No.

none

2.4.3 Type

liquid

2.4.4 Principal ingredients

Seawater

2.4.5 Temperature

14~27 °C

2.4.6 Salinity

> 32 psu

2.3.7 pH

6~8

2.4.8 Custody condition

Constant room at 4 °C for 7 days

2.4.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.4.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.4.11 Disposal of the resting substances

Scrap after test

2.4.12 Certificate of analysis

Client supplies to test organization

## 2.5 Preparation of the test substance (SOP#TP-011)

The concentration of test substance is diluted on the control seawater (non-treated ballast water) as crude liquid. The six concentrations of test substance including 0.00 % (only non-treated ballast water) and 100.00 % are arranged for the final definitive experiment. pH is not adjusted by  $8.0 \pm 0.2$  because we have assumed that acidification and/or alkalization of test water treated by BioViolet™ is also toxic effect.

## 2.6 Culture condition (SOP#TO-001)

### 2.6.1 Room

Incubator

### 2.6.2 Vessel type / volume

Glass flask / 250 mL

### 2.6.3 Medium volume / vessel volume & medium

100 mL/250 mL, f/2 (see Annex 2)

### 2.6.4 Inoculum density

$5 \times 10^3$  cells/mL

### 2.6.5 Temperature

20 °C

### 2.6.6 Interval of inoculum

Every a week

### 2.6.7 L:D cycle

Continuous light

### 2.6.8 Intensity of light

3,000 lux

## 2.7 Test system

### 2.7.1 Test organisms

- |                    |  |
|--------------------|--|
| .1 Scientific name | <i>Skeletonema costatum</i> (Strain number: VBA-007)         |
| .2 Producer        | Marine Eco-technology Institute Co., Ltd., Republic of Korea |
| .3 Dealer          | Marine Eco-technology Institute Co., Ltd., Republic of Korea |

### 2.7.2 Ground of selected test organisms

*Skeletonema costatum* is important as primary producer in trophic structure and widely distributed phytoplankton species (Phylum Bacillariophyta) in estuarine and coastal areas. It is used to evaluate the aquatic toxicity and has a large database.

### 2.7.3 Isolation of phytoplankton (SOP#TO-001)

Isolation method	Dilution techniques
Species origin	Yongho Bay in Korea

### 2.7.4 Preparation of pre-culture and inoculation (SOP#TP-008)

A pre-culture is started 2 to 4 days before the beginning of the experiment. In order to maintain the exponential growth phase until the start of the experiment, a pre-culture is made to inoculate  $1 \times 10^4$  cells/mL from the algal stock culture to the growth medium. The pre-culture is incubated under the same conditions as those in the experiment. And the pre-culture for the experiment with 3-32 psu treated ballast water is carried out same way after acclimation of test organisms for more than 14 days.

### 2.7.5 Check the test organisms for the experiment (SOP#TP-001)

The cell density in the pre-culture is immediately measured before use, in order to calculate the required inoculation volume and appearance of cells.

### 2.7.6 Disposal of the resting test organisms (SOP#TO-006)

The resting test organisms are scrapped after sterilization by the autoclave.

## 2.8 Study design

### 2.8.1 Test concentration (SOP#TP-011)

Control	Control seawater	100.00 %: Seawater treated by BioViolet™	0.00 %
6.25 %	Control seawater	93.75 %: Seawater treated by BioViolet™	6.25 %
12.50 %	Control seawater	87.50 %: Seawater treated by BioViolet™	12.50 %
25.00 %	Control seawater	75.00 %: Seawater treated by BioViolet™	25.00 %
50.00 %	Control seawater	50.00 %: Seawater treated by BioViolet™	50.00 %
100.00 %	Control seawater	0.00 %: Seawater treated by BioViolet™	100.00 %

### 2.8.2 Exposure conditions (SOP#TP-001)

The final cell density ( $3 \times 10^3$  cells/mL) is added from the algal pre-culture to the each concentration in the test vessels. And then the test vessels are incubated for 96 hours at  $22 \pm 1^\circ\text{C}$ .

Experiment conditions and criteria are shown Annex 3 and application site is shown Annex 5.

### 2.8.3 Observation item and measurement components

#### .1 Biomass (SOP#TP-009, SOP#TP-012)

Measurement of biomass is conducted by measuring chlorophyll *a* extracted with 90% acetone using Tuner Designs AU-10. The measurement of the chlorophyll *a* is immediately done within 2 hours after

extraction of it. To obtain the regression equation between chlorophyll *a* and cell density, cell count in the control is done by manual cell counting used the microscope (CKX 31, Olympus) with counting chamber.

Calculations of the growth and the inhibition growth rate are shown Annex 4.

#### .2 Other observations (SOP#TP-001)

Microscopic observation is performed to verify normal and healthy appearance of the inoculum culture and to observe any abnormal appearance of the algae at the end of the experiment.

#### .3 pH (SOP#TE-002)

pH of the solutions at the beginning and at the end of the experiment is measured.

### **2.9 Statistical procedures (SOP#SP-101, SOP#SP-102)**

For satisfactory correlation with biomass, chlorophyll *a* and cell density in the control are analyzed by linear correlation analysis with Excel program and the greatest  $R^2$  value is yielded. Cell density is calculated by equation from the linear correlation analysis.

NOEC, LOEC and EC50 values of the end point are estimated by statistical analysis procedure referred from USEPA (2002). TOXCALC 5.0 program (Tidepool scientific software, USA) is used for statistic analysis.

## **3. REFERENCES**

OECD guidelines for the testing of chemicals-201. 2006. Freshwater alga and Cyanobacteria, growth inhibition test. 25pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

## ANNEX 1

### Definitions

- 1. Coefficient of variation** is a dimensionless measure of the variability of a parameter, defined as the ratio of the standard deviation to the mean. This can also be expressed as a percent value. Mean coefficient of variation of average specific growth rate in replicate control cultures should be calculated as follows:

  1. Calculate % CV of average specific growth rate out of the daily/section by section growth rates for the respective replicate;
  2. Calculate the mean value out of all values calculated under point 1 to get the mean coefficient of variation of the daily/section by section specific growth rate in replicate control cultures.
- 2. EC<sub>x</sub>** is the concentration of the test substance dissolved in test medium that results in an x % (e.g. 50 %) reduction in growth of the test organisms within a stated exposure period.
- 3. Growth rate** (average specific growth rate) is the logarithmic increase in biomass during the exposure period.
- 4. Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant reducing effect on growth (at  $p < 0.05$ ) when compared with the control, within a given exposure time.
- 5. No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC.
- 6. Specific growth rate** is a response variable defined as quotient of the difference of the natural logarithms of a parameter of observation and the respective time period.
- 7. Pre-culture** is intended to give an amount of algae suitable for the inoculation of test cultures. The pre-culture is incubated under the conditions of the test and used when still exponentially growing, normally after an incubation period of 2 to 4 days.
- 8. Biomass** is the dry weight of living matter present in a population expressed in terms of a given volume; e.g., mg algae/litre test solution. Usually “biomass” is defined as a mass, but in this study this word is used to refer to mass per volume. Also in this study, surrogates for biomass as cell density (cells/mL) from fluorescence method.

## ANNEX 2

Composition of f/2 (Guillard & Ryther 1962, Guillard 1975)

Component	Stock solution	Quantity Used	Concentration in Final Medium (M)
NaNO <sub>3</sub>	75.0 g/L dH <sub>2</sub> O	1.0 mL	$8.82 \times 10^{-4}$
NaH <sub>2</sub> PO <sub>4</sub> ·4H <sub>2</sub> O	5.0 g/L dH <sub>2</sub> O	1.0 mL	$3.62 \times 10^{-5}$
Na <sub>2</sub> SiO <sub>3</sub> ·9H <sub>2</sub> O	30.0 g/L dH <sub>2</sub> O	2.0 mL	$1.06 \times 10^{-4}$
* f/2 Trace metal solution	(see recipe below)	1.0 mL	-
** f/2 Vitamin solution	(see recipe below)	0.5 mL	-
* f/2 Trace metal solution			
Component	Stock solution	Quantity Used	Concentration in Final Medium (M)
FeCl <sub>3</sub> ·6H <sub>2</sub> O	-	3.15 g	$1.17 \times 10^{-5}$
Na <sub>2</sub> EDTA·2H <sub>2</sub> O	-	4.36 g	$1.17 \times 10^{-5}$
CuSO <sub>4</sub> ·5H <sub>2</sub> O	9.8 g/L dH <sub>2</sub> O	1.0 mL	$3.93 \times 10^{-8}$
Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	6.3 g/L dH <sub>2</sub> O	1.0 mL	$2.60 \times 10^{-8}$
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	22.0 g/L dH <sub>2</sub> O	1.0 mL	$7.65 \times 10^{-8}$
CoCl <sub>2</sub> ·6H <sub>2</sub> O	10.0 g/L dH <sub>2</sub> O	1.0 mL	$4.20 \times 10^{-8}$
MnCl <sub>2</sub> ·4H <sub>2</sub> O	180.0 g/L dH <sub>2</sub> O	1.0 mL	$9.10 \times 10^{-7}$
** f/2 Vitamin solution			
Component	Stock solution	Quantity Used	Concentration in Final Medium (M)
Cyanocobalamin (Vitamin B <sub>12</sub> )	1.0 g/L dH <sub>2</sub> O	1.0 mL	$3.69 \times 10^{-10}$
Biotin (Vitamin H)	0.1 g/L dH <sub>2</sub> O	10.0 mL	$2.05 \times 10^{-9}$
Thiamine HCl (vitamin B <sub>1</sub> )	-	200.0 mL	$2.96 \times 10^{-7}$

### ANNEX 3

Experiment conditions and acceptability criteria for diatom, *Skeletonema costatum*, chronic growth inhibition tests with the > 32 and 3-32 psu de-ballast water from the BioViolet™

Test parameter	Conditions
Test type	Static
Temperature	22 ± 1 °C
Light quality	“Cool white” fluorescent lamp
Light intensity	3,000 lux
Photoperiod	Continuous illumination
Test chamber size	15 mL
Test solution volume	10 mL
Renewal of test solutions	None
Initial cell density in test chambers	3,000 cells/mL
No. replicate chambers per concentration	3
Shaking rate	Twice daily by hand
Aeration	None
Dilution water	Filtered seawater (Control seawater) by 0.2µm membrane filter
Test concentrations	Effluents: 6 including receiving water 100.00% and a control
Test dilution factor	Effluents: 0.5
Test duration	96 hours
Endpoint	Growth inhibition
Test acceptability criteria	Specific growth rate in the control: $\geq 0.92 \text{ day}^{-1}$ Variation coefficient of the control specific growth rate: $\leq 7\%$



## ANNEX 4

1. The average specific growth rate for a specific period is calculated from equation [1]:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} (\text{day}^{-1}) \quad [1]$$

where:

$\mu_{i-j}$  is the average specific growth rate from time I to j;

$X_i$  is the biomass at time  $i$ ;

$X_j$  is the biomass at time  $j$ ;

2. The percent inhibition of growth rate for each treatment replicate is calculated from equation [2]:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100 \quad [2]$$

where:

$\%I_r$  is percent inhibition in average specific growth rate;

$\mu_C$  is mean value for average specific growth rate ( $\mu$ ) in the control group ;

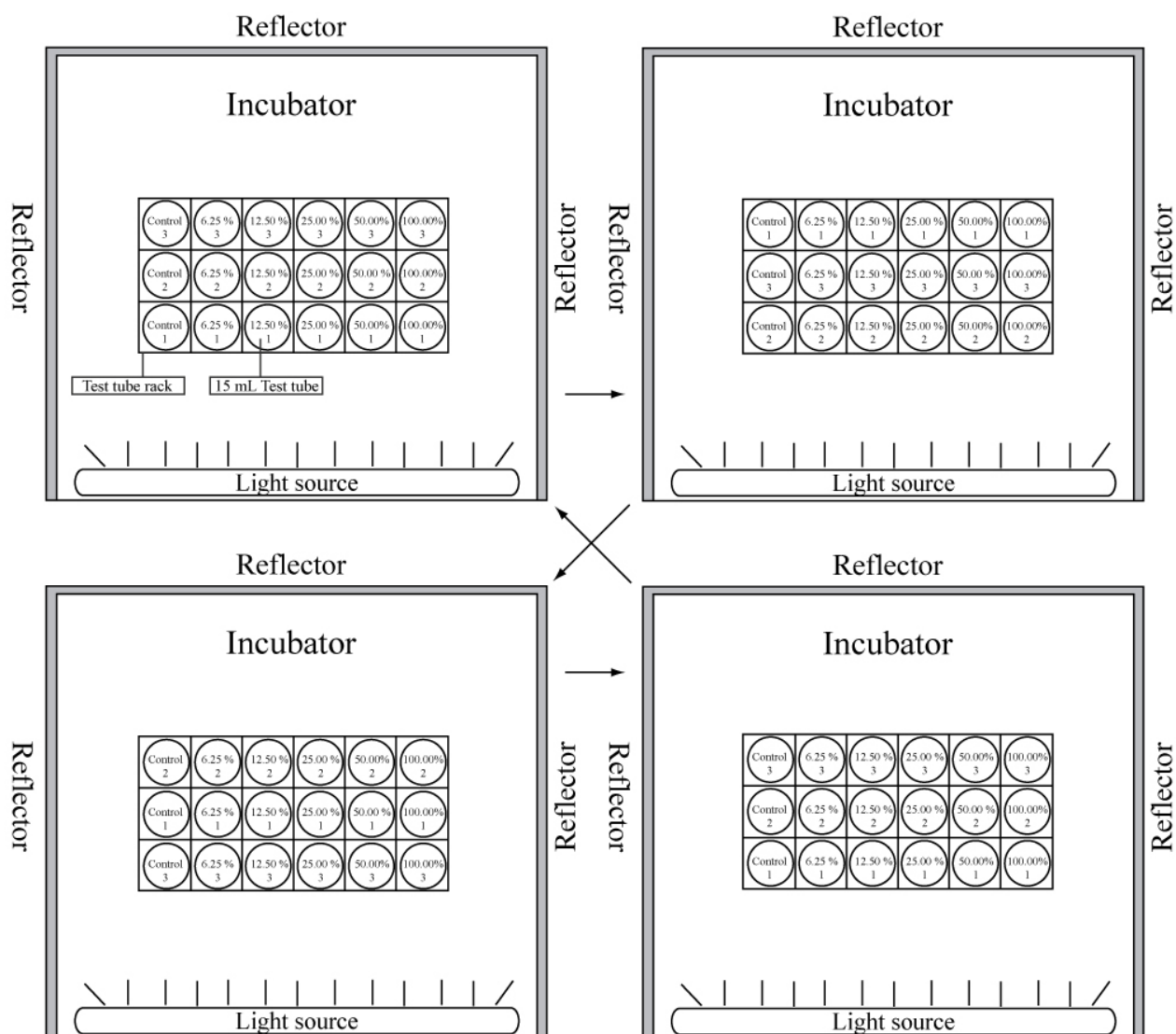
$\mu_T$  is average specific growth rate ( $\mu$ ) in treatment replicate

Each treatment group and control group are calculated a mean value for growth rate along with variance estimates.

## ANNEX 5

### Application Site

Application site for the chronic toxicity test with diatom, *Skeletonema costatum* is shown as follow. The arrowheads indicate the systematical rotation of the test vessels.



## **2.2 Acute Toxicity Test**

### **2.2.1 ANNEX 1**

***Brachionus plicatilis*, Acute Toxicity Test**



Marine Eco-technology Institute Co., Ltd.  
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# STUDY PLAN

**Acute Aquatic Toxicity For > 32 & 3-32 psu Treated Ballast Water of  
BioViolet™**

**- Rotifer, *Brachionus plicatilis* Acute Toxicity Test**

**Study Name: BioViolet™**

**Study No: BW- DBWT1107-KS**

Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## STUDY PLAN PREPARATION AND APPROVAL

Study title: Acute toxicity test to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioViolet™ toward survival of *Brachionus plicatilis*

Study name: BioViolet™ Study No: BW-DBWT1107-KS

Test facility Marine Eco-technology Institute Co., Ltd.

2011. 02. 28.

Study Director: Sang-Hee Shin

(signature)

Lab. Manager: Myung-Baek Shon

(signature)

Project Officer: Min Ho Son

(signature)

Organization Korean Marine Equipment Research Institute

2011. 02. 28.

Test Facility Manager: Jun-Hak Lee

(signature)

Project Director: Young-Soo Kim

(signature)

Kwang San Co., Ltd.

2011. 02. 28.

Monitor: Sang Bum Bae

(signature)

Sponsor: Young Woo Lee

(signature)

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# **1. OUTLINE OF STUDY**

## **1.1 Purpose**

The purpose of this study is to assess the acute toxic effects of the > 32 and 3-32 psu ballast water treated by the BioViolet™ (de-ballast water from the BWMS) on the survival of *Brachionus plicatilis*.

## **1.2 Principle**

Neonate of *Brachionus plicatilis* aged less than 2 hours at the start of test is exposed to the treated ballast water of the BioViolet™ at a range of concentrations including 100.00 % effluent, for a period of 24 hours. Mortality are recorded at 24 hours and compared with control values. The results are analyzed in order to calculate the LC50 at 24 hours.

## **1.3 Good Laboratory Practice**

All procedure of this study is complied with following GLP regulation:

- “OECD Principles of Good Laboratory Practice”

Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

## **1.4 Guideline**

This study using *Brachionus plicatilis* was complied with principle and conducted on the basis of following standard method:

- “Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus*. E 1440-91”

American Society of Testing Materials, (Reapproved 2004)

## **1.5 Sponsor**

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

Tel: +82-51-974-6351

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## **1.6 Test facility**

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

Tel: + 82-51-611-6200

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## 1.7 Test site

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## 1.8 Study director

Name: Sang-Hee Shin

Department: 2<sup>nd</sup> research team

## 1.9 Study timetable

### 1.9.1 Treated ballast water (3-32 psu) and non-Treated ballast water (3-32 psu)

Study initiation	2011. 02. 28
Reference toxicant test	2011. 08. 08 ~ 08. 09
Induced to hatch for cysts	2011. 09. 26
Separation of neonates for test	2011. 09. 27
Collect of sample	2011. 09. 26
Experimental start	2011. 09. 27
Exposure date	2011. 09. 27
Evaluation survival	2011. 09. 28
Experimental completion	2011. 09. 28
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### 1.9.2 Treated ballast water (> 32 psu) and non-Treated ballast water (> 32 psu)

Study initiation	2011. 02. 28
Induced to hatch for cysts	2011. 11. 07
Separation of neonates for test	2011. 11. 08
Collect of sample	2011. 11. 07
Experimental start	2011. 11. 08
Exposure date	2011. 11. 08
Evaluation survival	2011. 11. 09
Experimental completion	2011. 11. 09
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30



Study completion

2011. 12. 31

### **1.10 Responsible Personnel**

Laboratory manager (Test site manager)	Myung-Baek Shon
Statistical analysis	
Culture of test organisms	Tae Won Kim
Observation and record	
Sampling, transit and custody of test substance or solution	
Disposal of the resting substance	Je Kwan Park
Check the chemical condition	
Disposal of the resting organisms	Hyeong Ju Seok
Sampling, transit and custody of test substance or solution	

### **1.11 Retention of records and data**

#### 1.11.1 Duration

Duration of storage is 5 years after end of the study. It will be decided that storage of data after 5 years should be consulted with the client.

#### 1.11.2 Place of storage

Name: Archives of Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

#### 1.11.3 Retention of records and data

Study plan, final report, raw data, documents related with the study, communication documents, and etc.

## **2. MATERIALS AND METHODS**

### **2.1 Test substance-1 (Treated ballast water (3-32 psu) -Treated)**

2.1.1 Substance name

Treated water

2.1.2 Lot No.

none

2.1.3 Type

liquid

2.1.4 Principal ingredients

Seawater

2.1.5 Temperature

14~27 °C

2.1.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.1.8 Custody condition

Constant room at 4 °C for 7 days

2.1.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.1.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.1.11 Disposal of the resting substances

Scrap after test

2.1.12 Certificate of analysis

Client supplies to test organization

## **2.2 Test substance-2 (Non-treated ballast water (3-32 psu) -Control)**

2.2.1 Substance name

Non-treated water

2.2.2 Lot No.

none

2.2.3 Type

liquid

2.2.4 Principal ingredients

Seawater

2.2.5 Temperature

14~27 °C

2.2.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.2.8 Custody condition

Constant room at 4 °C for 7 days

2.2.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.2.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.2.11 Disposal of the resting substances

Scrap after test

2.2.12 Certificate of analysis

Client supplies to test organization

### **2.3 Test substance-3 (Treated ballast water (> 32 psu) -Treated)**

2.3.1 Substance name

Treated water

2.3.2 Lot No.

none

2.3.3 Type

liquid

2.3.4 Principal ingredients

Seawater

2.3.5 Temperature

14~27 °C

2.3.6 Salinity

> 32 psu

2.3.7 pH

6~8

2.3.8 Custody condition

Constant room at 4 °C for 7 days

2.3.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.3.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.3.11 Disposal of the resting substances

Scrap after test

2.3.12 Certificate of analysis

Client supplies to test organization

## **2.4 Test substance-4 (Non-treated ballast water (> 32 psu) -Control)**

2.4.1 Substance name

Non-treated water

2.4.2 Lot No.

none

2.4.3 Type

liquid

2.4.4 Principal ingredients

Seawater

2.4.5 Temperature

14~27 °C

2.4.6 Salinity

> 32 psu

2.3.7 pH

6~8

2.4.8 Custody condition

Constant room at 4 °C for 7 days

2.4.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.4.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.4.11 Disposal of the resting substances

Scrap after test

2.4.12 Certificate of analysis

Client supplies to test organization

## 2.5 Preparation of the test substance (SOP#TP-011)

The concentration of test substance is diluted on the control seawater (non-treated ballast water) as crude liquid. The six concentrations of test substance including 0.00 % (only non-treated ballast water) and 100.00 % are arranged for the final definitive experiment.

## 2.6 Culture condition

### 2.6.1 Hatching of test organisms (SOP#TO-007)

Test organisms	<i>Brachionus plicatilis</i>
Vessel	6 hole plate
Medium	20 psu filtered seawater (0.45µm membrane filter, Whatman)
Temperature	25 °C
Light source	“cool-white” fluorescent lamp
Light cycle	Continuous light
Light cycle	1,000 ~ 3,000 lux

The cysts were obtained from commercial supplier (See ‘2.7 Test system’).

### 2.6.2 Feeding regime

None

## 2.7 Test system

### 2.7.1 Test organisms

.1 Scientific name	<i>Brachionus plicatilis</i>
.2 Producer	MicroBioTests Inc., Belgium
.3 Dealer	Marine Eco-technology Institute Co., Ltd., Republic of Korea

### 2.7.2 Ground of selected test organisms

Marine rotifer, *Brachionus plicatilis* have the advantages of cosmopolitan distribution, wide salinity adaptability, important of trophic roles as primary consumer in marine ecosystem, short generation time and easy culture in small spaces. For this reason *Brachionus plicatilis* is commonly used as a toxicity test.

### 2.7.3 Preparation of the test organisms (SOP#TO-007)

Hatching rotifer cyst is initiated approximately 24 h prior to the start of the test, and have to be carried out in seawater of reduced salinity (20 psu). They are incubated at 25 °C and 1,000 to 3,000 lux continuous illumination. They are checked the hatching hourly after 24 h incubation in order to collect the test animals

within 2 hours of their hatching. For the > 32 psu de-ballast water, rotifer neonates are acclimated for approximately 1 hour to adapt to the eventual salinity change.

#### 2.7.4 Check the test organisms for the test (SOP#TO-007)

Health of the test organisms such as the mortality, appearance and abnormal behavior is observed with a microscope (SZ51, Olympus).

#### 2.7.5 Disposal of the resting test organisms (SOP#TO-006)

The resting test organisms are scrapped after sterilization by the autoclave.

## 2.8 Study design

#### 2.8.1 Test concentration (SOP#TP-011)

Control	Control seawater	100.00 %: Seawater treated by BioViolet™	0.00 %
6.25 %	Control seawater	93.75 %: Seawater treated by BioViolet™	6.25 %
12.50 %	Control seawater	87.50 %: Seawater treated by BioViolet™	12.50 %
25.00 %	Control seawater	75.00 %: Seawater treated by BioViolet™	25.00 %
50.00 %	Control seawater	50.00 %: Seawater treated by BioViolet™	50.00 %
100.00 %	Control seawater	0.00 %: Seawater treated by BioViolet™	100.00 %

#### 2.8.2 Exposure conditions (SOP#TP-013)

*Brachionus plicatilis* is placed in test vessels divided into five neonates each replication of concentration including the control. And then the test vessels are placed in incubator for 24 hours at  $25 \pm 1^\circ\text{C}$ .

Experiment conditions and criteria are shown Annex 2 and application site is shown Annex 3.

#### 2.8.3 Observation item and measurement components

##### .1 Mortality (SOP#TP-013)

Each test vessel is checked for mortality of *Brachionus plicatilis* at 24 hours after the beginning of the experiment. Justification of mortality is that animals do not exhibit any internal or external movement in 5 seconds of observation.

##### .2 Other observations (SOP#TP-013)

In addition to mortality, any abnormal behavior and appearance are recorded.

##### .3 Temperature (SOP#TE-002)

Temperature of the solutions is measured at the beginning and end of the experiment.

.4 Dissolved oxygen (SOP#TE-002)

Dissolved oxygen (DO) of the solutions is measured at the beginning of the experiment.

(Test vessels contain only 1 mL, it is technically difficult to measure DO at the end of the experiment. It is also known that brachionid rotifers are not sensitive to low oxygen levels.)

.5 pH (SOP#TE-002)

pH of the solutions is measured at the beginning and end of the experiment.

## **2.9 Reference toxicant test**

.1 Test substance: potassium dichromate (CAS No.: 7778509)

.2 The test procedure and condition

The test procedure and condition of reference toxicant test is same to '2.7 Test system' '2.8.2 Exposure conditions' and '2.8.3 Observation item and measurement component' in this study.

.3 Concentrations: Control, 100, 180, 320, 560, 1000 mg/L

.4 Precision objects (see Annex 2)

End point: 24hour mortality (24h-LC50)

Mean (Standard deviation) value: 384.4 ( $\pm 175.4$ ) mg/L

## **2.10 Statistical procedures (SOP#SP-101)**

LC50 values of the end point are estimated by statistical analysis procedure referred from USEPA (2002). TOXCALC 5.0 program (Tidepool scientific software, USA) is used for statistic analysis.

## **3. REFERENCES**

ASTM. 2004. Standard guide for acute toxicity test with the rotifer *Brachionus*. E-1440-91. 8pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.



## **ANNEX 1**

### **Definitions**

**1. Rotifer cyst** is that a rotifer embryo arrested at an early stage in development, enclosed in an envelope and resistant to desiccation and temperature extremes. Rotifer cysts are often incorrectly referred to as resting eggs. Upon hydration, embryonic development resumes until a neonate female emerges from the cyst.

**2. Rotifer neonate** is a newly hatched, freely swimming rotifer. All neonates hatched from cysts are females.

**3. LCx (Lethal concentration)** is the concentration estimated to die x percent of the test organisms within a stated exposure period.

## ANNEX 2

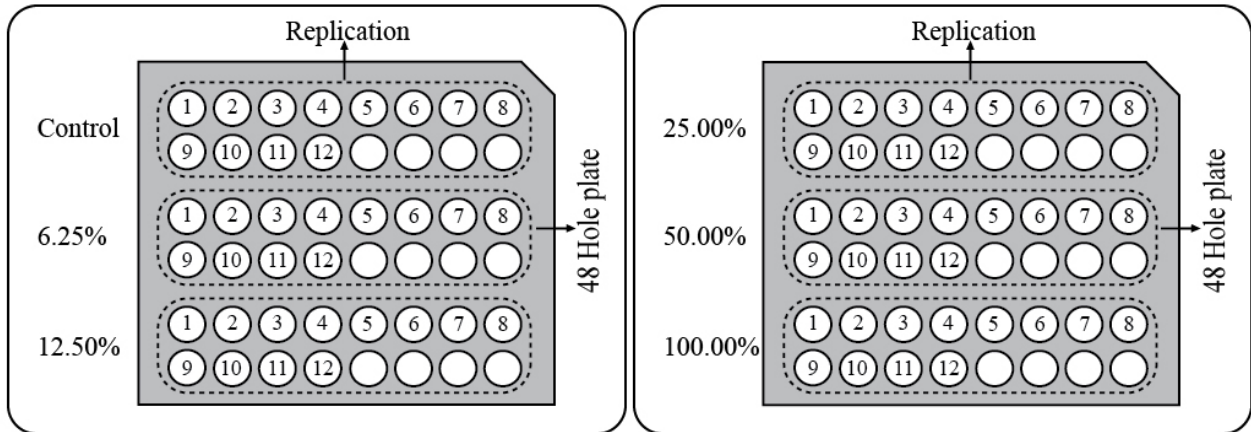
Experiment conditions and acceptability criteria for the rotifer, *Brachionus plicatilis* acute immobilization and survival toxicity tests with the > 32 and 3-32 psu de-ballast water from the BioViolet™

Test parameter	Conditions
Test type	Static (non-renewal)
Temperature	25 ± 1 °C
Light quality	None
Light intensity	Darkness
Photoperiod	0h light: 24 h dark
Test chamber size	1.5 mL (48 hole plate)
Test solution volume	1mL
Renewal of test solutions	None
Age of test organisms	Less than 2 hours
Number of individuals per test chamber	5 inds.
Replicate test chambers per concentration	12
Feeding regime	None
Cleaning	None
Aeration	None
Dilution water	Filtered seawater (Control seawater) by 0.45 µm membrane filter
Test concentration	5 including 100.00% effluents and a control
Dilution factor	Effluents: 0.5
Test duration	24 hours
Endpoints	survival
Test acceptability criteria	90% or greater survival of all control organisms
Precision object	Reference toxicant: potassium dichromate 24h LC50 value: 209.0~559.9 mg/L

## ANNEX 3

### Application Site

Application site for the acute toxicity test with the rotifer, *Brachionus plicatilis* is shown as follow.



### **2.2.2 ANNEX 2**

**The olive flounder fish, *Paralichthys olivaceus*, Acute Toxicity Test**



Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea  
Tel +82-51-611-6200  
Fax +82-51-611-0588

# STUDY PLAN

**Acute Aquatic Toxicity For > 32 & 3-32 psu Treated Ballast Water of BioViolet™**

**- The olive flounder fish, *Paralichthys olivaceus*, Acute Toxicity Test**

**Study Name: BioViolet™**

**Study No: BW- DBWT1107-KS**

Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## STUDY PLAN PREPARATION AND APPROVAL

Study title: Acute toxicity to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioViolet™ toward survival of olive flounder fish, *Paralichthys olivaceus*

Study name: BioViolet™ Study No: BW-DBWT1107-KS

Test facility Marine Eco-technology Institute Co., Ltd.

2011. 02. 28.

Study Director: Jin Hee Kim

(signature)

Lab. Manager: Myung-Baek Shon

(signature)

Project Officer: Min Ho Son

(signature)

Organization Korean Marine Equipment Research Institute

2011. 02. 28.

Test Facility Manager: Jun-Hak Lee

(signature)

Project Director: Young-Soo Kim

(signature)

Kwang San Co., Ltd.

2011. 02. 28.

Monitor: Sang Bum Bae

(signature)

Sponsor: Young Woo Lee

(signature)

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# **1. OUTLINE OF STUDY**

## **1.1 Purpose**

The purpose of this study is to assess the acute toxic effects of the > 32 and 3-32 psu ballast water treated by the BioViolet™ (de-ballast water from the BWMS) on the survival of juvenile olive flounder, *Paralichthys olivaceus*.

## **1.2 Principle**

The juvenile olive flounder, *Paralichthys olivaceus* is exposed to the treated ballast water of the BioViolet™ for a period of 96 hours. Mortalities are recorded at 24, 48, 72 and 96 hours and the concentration which kills 50 percent of the fish (LC50) are determined where possible.

## **1.3 Good Laboratory Practice**

All procedure of this study is complied with following GLP regulation:

- “OECD Principles of Good Laboratory Practice”

Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

## **1.4 Guideline**

This study is conducted on the basis of principle of following standard method and applied for *Paralichthys olivaceus*:

- “OECD Guideline for Testing of Chemicals, 203, Fish, Acute Toxicity Test”

Organization for Economic Co-operation and Development, (Adopted: July. 17, 1992)

## **1.5 Sponsor**

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

Tel: +82-51-974-6351

Fax: +82-51-974-6405

## **1.6 Test facility**

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

Tel: + 82-51-611- 6200

Fax: + 82-51-611- 0588



## 1.7 Test site

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## 1.8 Study Director

Name: Jin Hee Kim

Department: 3<sup>rd</sup> research team

## 1.9 Study timetable

### 1.9.1 Treated ballast water (3-32 psu) and non-Treated ballast water (3-32 psu)

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 08. 14
Acclimatization on Lab Condition (20 psu)	2011. 08. 14 ~ 09. 04
Settling in the test vessel	2011. 09. 04
Observation of mortality	2011. 09. 04 ~ 09. 06
Collect of sample	2011. 09. 06
Experimental start	2011. 09. 07
Exposure date	2011. 09. 07
Evaluation of mortality	2011. 09. 07 ~ 09. 11
Experimental completion	2011. 09. 11
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### 1.9.2 Treated ballast water (> 32 psu) and non-Treated ballast water (> 32 psu)

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 10. 15
Acclimatization on Lab Condition (34 psu)	2011. 10. 15 ~ 10. 29
Settling in the test vessel	2011. 10. 29
Observation of mortality	2011. 10. 29 ~ 10. 31
Collect of sample	2011. 10. 31
Experimental start	2011. 11. 01
Exposure date	2011. 11. 01

Evaluation of mortality	2011. 11. 01 ~ 11. 05
Experimental completion	2011. 11. 05
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### **1.10 Responsible Personnel**

Laboratory manager (Test site manager)	Myung-Baek Shon
Statistical analysis	
Culture of test organisms	Tae Won Kim
Observation and record	
Sampling, transit and custody of test substance or solution	
Disposal of the resting substance	Je Kwan Park
Check the chemical condition	
Disposal of the resting organisms	Hyeong Ju Seok
Sampling, transit and custody of test substance or solution	

### **1.11 Retention of records and data**

#### **1.11.1 Duration**

Duration of storage is 5 years after end of the study. It will be decided that storage of data after 5 years should be consulted with the client.

#### **1.11.2 Place of storage**

Name: Archives of Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

#### **1.11.3 Retention of records and data**

Study plan, final report, raw data, documents related with the study, communication documents, and etc.

## 2. MATERIALS AND METHODS

### 2.1 Test substance-1 (Treated ballast water (3-32 psu) -Treated)

2.1.1 Substance name

Treated water

2.1.2 Lot No.

none

2.1.3 Type

liquid

2.1.4 Principal ingredients

Seawater

2.1.5 Temperature

14~27 °C

2.1.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.1.8 Custody condition

Constant room at 4 °C for 7 days

2.1.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.1.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.1.11 Disposal of the resting substances

Scrap after test

2.1.12 Certificate of analysis

Client supplies to test organization

## **2.2 Test substance-2 (Non-treated ballast water (3-32 psu) -Control)**

2.2.1 Substance name

Non-treated water

2.2.2 Lot No.

none

2.2.3 Type

liquid

2.2.4 Principal ingredients

Seawater

2.2.5 Temperature

14~27 °C

2.2.6 Salinity

3-32 psu

2.2.7 pH

6~8

2.2.8 Custody condition

Constant room at 4 °C for 7 days

2.2.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.2.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.2.11 Disposal of the resting substances

Scrap after test

2.2.12 Certificate of analysis

Client supplies to test organization

### **2.3 Test substance-3 (Treated ballast water (> 32 psu) -Treated)**

2.3.1 Substance name

Treated water

2.3.2 Lot No.

none

2.3.3 Type

liquid

2.3.4 Principal ingredients

Seawater

2.3.5 Temperature

14~27 °C

2.3.6 Salinity

3-32 psu

2.3.7 pH

6~8

2.3.8 Custody condition

Constant room at 4 °C for 7 days

2.3.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.3.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.3.11 Disposal of the resting substances

Scrap after test

2.3.12 Certificate of analysis

Client supplies to test organization

## **2.4 Test substance-4 (Non-treated ballast water (> 32 psu) -Control)**

2.4.1 Substance name

Non-treated water

2.4.2 Lot No.

none

2.4.3 Type

liquid

2.4.4 Principal ingredients

Seawater

2.4.5 Temperature

14~27 °C

2.4.6 Salinity

> 32 psu

2.4.7 pH

6~8

2.4.8 Custody condition

Constant room at 4 °C for 7 days

2.4.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.4.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.4.11 Disposal of the resting substances

Scrap after test

2.4.12 Certificate of analysis

Client supplies to test organization

## 2.5 Preparation of the test substance (SOP#TP-011)

The concentration of test substance is diluted on the control seawater (non-treated ballast water) as crude liquid. The six concentrations of test substance including 0.00 % (only non-treated ballast water) and 100.00 % are arranged for the final definitive experiment.

## 2.6 Culture condition (SOP#TO-005)

### 2.6.1 Holding of test organisms

Test organisms	<i>Paralichthys olivaceus</i>
Culture type	Semi- recycle filter system
Vessel	1,000 L cubic aquarium
Medium	600 L filtered seawater (1µm CP Filter, Chisso Filter)
Temperature	20 ± 1 °C
Light source	“cool-white” fluorescent lamp
Light cycle	L : D = 16 h : 8 h
Light intensity	1,000 lux
Dissolved oxygen	6.0~8.0 mg/L

### 2.6.2 Feeding regime

Type of diet	Su hyup feed for fish 1-1S
Supplier	National Federation of Fisheries Cooperatives
Diet supply period	once a day

## 2.7 Test system

### 2.7.1 Test organisms

.1 Scientific name	<i>Paralichthys olivaceus</i>
.2 Producer	KyeongYang Fisheries, Yo-Su city, Jeolla province, Republic of Korea
.3 Dealer	Marine Eco-technology Institute Co., Ltd., Republic of Korea

### 2.7.2 Ground of selected test organisms

Pleuronectiformes Paralichthyidae *Paralichthys olivaceus* called olive flounder is widely distributed in coastal water of Korea and is important as economic marine products. *P. olivaceus* plays in marine trophic structure as a top predator.

### 2.7.3 Preparation of the test organisms (SOP#TP-006)

*Paralichthys olivaceus* aged 30 ~ 50 days is acclimated under the laboratory and test condition for two or three weeks until 24 hours before the beginning of the experiment. Following a 48 hours settling-in period, mortality is recorded and the following criteria applied:

1. Mortality of greater than 10 percent of population in seven days: rejection of entire batch.
2. Mortality of between 5 and 10 percent of population: acclimatization continued for seven additional 7 days.
3. Mortality of less than 5 percent of population: acceptance of batch.

### 2.7.4 Check the test organisms for the experiment (SOP#TP-006)

Health of the test organisms such as the mortality, appearance and abnormal behavior is observed.

### 2.7.5 Disposal of the remaining test organisms (SOP#TO-006)

The resting test organisms are euthanized under tricaine methanesulfonate (MS-222).

## 2.8 Study design

### 2.8.1 Test concentration (SOP#TP-011)

Control	Control seawater	100.00 %: Seawater treated by BioViolet™	0.00 %
6.25 %	Control seawater	93.75 %: Seawater treated by BioViolet™	6.25 %
12.50 %	Control seawater	87.50 %: Seawater treated by BioViolet™	12.50 %
25.00 %	Control seawater	75.00 %: Seawater treated by BioViolet™	25.00 %
50.00 %	Control seawater	50.00 %: Seawater treated by BioViolet™	50.00 %
100.00 %	Control seawater	0.00 %: Seawater treated by BioViolet™	100.00 %

### 2.8.2 Exposure conditions (SOP#TP-006)

20 fish of *Paralichthys olivaceus* on the > 32 and 3-32 psu treated ballast water is placed in the each replication of test vessel. The test vessels are placed in the constant room for 96 hours at  $20 \pm 1$  °C.

Experiment conditions and criteria are shown Annex 2 and application site is shown Annex 3.

### 2.8.3 Observation item and measurement components

#### .1 Mortality (SOP#TP-006)

Fish will be considered dead if there is no visible movement and if touching of the caudal peduncle produces no reaction. Mortalities are recorded and then dead animals are removed.

#### .2 Other observations (SOP#TP-006)

Records are kept of visible abnormalities, such as loss of equilibrium, swimming behavior,



respiratory function and pigmentation.

.3 Temperature (SOP#TE-002)

Temperature of the solutions on each vessel during the experiment period is measured daily.

.4 Dissolved oxygen (SOP#TE-002)

DO of the solutions on each vessel during the experiment period is measured daily.

.5 pH (SOP#TE-002)

pH of the solutions on each vessel during the experiment period is measured daily.

## **2.9 Statistical procedures (SOP#SP-101)**

LC25 and LC50 values of the end point are estimated by statistical analysis procedure referred from USEPA (2002). TOXCALC 5.0 program (Tidepool scientific software, USA) is used for statistic analysis.

## **3. REFERENCES**

OECD guidelines for the testing of chemicals-203. 1992. Fish, Acute toxicity test. 9 pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

## **ANNEX 1**

### **Definitions**

**1. Semi-static test** is a test without flow of solution, but with occasional batch wise renewal of the test solution after prolonged periods.

**2. LC25 and LC50** in this study is the median lethal concentration, i.e. that concentration of the test substance in water which kills 25 percent and 50 percent of a test batch of fish within a particular period of exposure.

## ANNEX 2

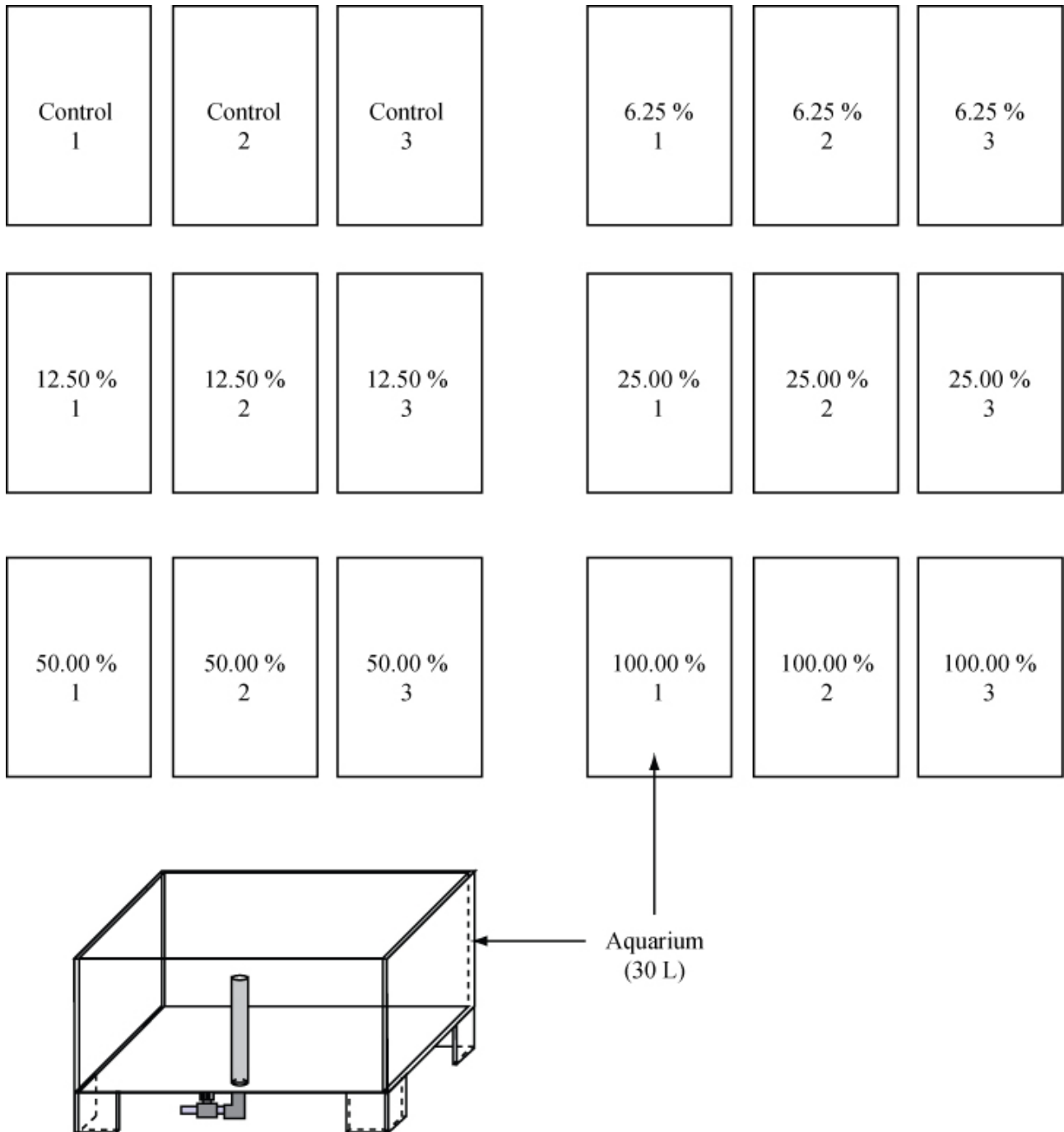
Experiment conditions and acceptability criteria for olive flounder, *Paralichthys olivaceus*, acute mortality toxicity tests with the > 32 and 3-32 psu de-ballast water from the BioViolet™

Test parameter	Conditions
Test type	Semi-static
Temperature	20±1 °C
Light quality	“Cool white” fluorescent lighting
Light intensity	1,000 lux
Photoperiod	16 h light: 8 h dark
Test chamber size	30 L
Test solution volume	20 L
Renewal of test solutions	Every other day
Age of test organisms	Less than 50 days old
Number of individuals per test chamber	20
Replicate test chambers per concentration	3
Feeding regime	None
Cleaning	None
Aeration	To maintain DO (> 60 % of the air saturation value)
Dilution water	Filtered seawater (Control seawater) by 1µm CP Filter, Chisso Filter
Test concentration	5 including 100.00% effluents and a control
Dilution factor	Effluents: 0.5
Test duration	96 hours
Endpoints	Survival
Test acceptability criteria	90% or greater survival of all control organisms

## ANNEX 3

### Application Site

Application site for the acute toxicity test with olive flounder, *Paralichthys olivaceus* is shown as follow.



## **2.3 Chronic Toxicity Test**

### **2.3.1 ANNEX 1**

***Brachionus plicatilis*, Chronic Toxicity Test**



Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea  
Tel +82-51-611-6200  
Fax +82-51-611-0588

# STUDY PLAN

**Chronic Aquatic Toxicity For > 32 & 3-32 psu Treated Ballast Water of BioViolet™**

**- Rotifer, *Brachionus plicatilis*, Chronic Toxicity Test**

**Study Name: BioViolet™**

**Study No: BW- DBWT1107-KS**

Marine Eco-technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## STUDY PLAN PREPARATION AND APPROVAL

Study title: Chronic toxicity test to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioViolet™ toward population growth rate of *Brachionus plicatilis*

Study name: BioViolet™ Study No: BW-DBWT1107-KS

Test facility Marine Eco-technology Institute Co., Ltd.

2011. 02. 28.

Study Director: Sang-Hee Shin

(signature)

Lab. Manager: Myung-Baek Shon

(signature)

Project Officer: Min Ho Son

(signature)

Organization Korean Marine Equipment Research Institute

2011. 02. 28.

Test Facility Manager: Jun-Hak Lee

(signature)

Project Director: Young-Soo Kim

(signature)

Kwang San Co., Ltd.

2011. 02. 28.

Monitor: Sang Bum Bae

(signature)

Sponsor: Young Woo Lee

(signature)

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# **1. OUTLINE OF STUDY**

## **1.1 Purpose**

The purpose of this study is to assess the chronic toxic effects of the > 32 and 3-32 psu ballast water treated by the BioViolet™ (de-ballast water from the BWMS) on the population growth rate of *Brachionus plicatilis*.

## **1.2 Principle**

Neonate of *Brachionus plicatilis* aged less than 2 hours at the start of test is exposed to the treated ballast water of the BioViolet™ at a range of concentrations including 100.00% effluent, for a period of 96 hours. The rotifer density was counted every 24 hours until 96 hours. At the end of the experiment, the population growth rate ( $r$ ) is calculated that count the number of live rotifers in each test concentration compared with control values. And the percentage inhibition ( $I\%$ ) of the rotifer growth for each test concentration is evaluated. The results are analyzed in order to calculate the NOEC, LOEC and EC50 at 96 hours.

## **1.3 Good Laboratory Practice**

All procedure of this study is complied with following GLP regulation:

- “OECD Principles of Good Laboratory Practice”

Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

## **1.4 Guideline**

This study is conducted on the basis of principle of following standard method and applied for *Brachionus plicatilis*:

- “Cyst-based toxicity tests. VIII. Short-chronic toxicity tests with the freshwater rotifer *Brachionus calyciflorus*” (Janssen et al., 1994)

The test preparation in this study is complied on the basis of following standard method:

- “Standard Guide for Acute Toxicity Test with the Rotifer *Brachionus*. E 1440-91”  
American Society of Testing Materials, (Reapproved 2004)

## **1.5 Sponsor**

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

Tel: +82-51-974-6351

Fax: +82-51-974-6405

## 1.6 Test facility

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

Tel: + 82-51-611-6200

Fax + 82-51-611-0588

## 1.7 Test site

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## 1.8 Study director

Name: Sang-Hee Shin

Department: 2<sup>nd</sup> research team

## 1.9 Study timetable

### 1.9.1 Treated ballast water (3-32 psu) and non-Treated ballast water (3-32 psu)

Study initiation	2011. 02. 28
Induced to hatch for cysts	2011. 09. 26
Separation of neonates for test	2011. 09. 27
Collect of sample	2011. 09. 26
Experimental start	2011. 09. 27
Exposure date	2011. 09. 27
Evaluation population growth rate	2011. 09. 27 ~ 09. 28
Experimental completion	2011. 09. 28
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### 1.9.2 Treated ballast water (> 32 psu) and non-Treated ballast water (> 32 psu)

Study initiation	2011. 02. 28
Induced to hatch for cysts	2011. 11. 07
Separation of neonates for test	2011. 11. 08
Collect of sample	2011. 11. 07
Experimental start	2011. 11. 08

Exposure date	2011. 11. 08
Evaluation population growth rate	2011. 11. 08 ~ 11. 09
Experimental completion	2011. 11. 09
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### **1.10 Responsible Personnel**

Laboratory manager (Test site manager)	Myung-Baek Shon
Statistical analysis	
Culture of test organisms	Tae Won Kim
Observation and record	
Sampling, transit and custody of test substance or solution	
Disposal of the resting substance	Je Kwan Park
Check the chemical condition	
Disposal of the resting organisms	Hyeong Ju Seok
Sampling, transit and custody of test substance or solution	

### **1.11 Retention of records and data**

#### **1.11.1 Duration**

Duration of storage is 5 years after end of the study. It will be decided that storage of data after 5 years should be consulted with the client.

#### **1.11.2 Place of storage**

Name: Archives of Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

#### **1.11.3 Retention of records and data**

Study plan, final report, raw data, documents related with the study, communication documents, and etc.

## **2. MATERIALS AND METHODS**

### **2.1 Test substance-1 (Treated ballast water (3-32 psu) -Treated)**

2.1.1 Substance name

Treated water

2.1.2 Lot No.

none

2.1.3 Type

liquid

2.1.4 Principal ingredients

Seawater

2.1.5 Temperature

14~27 °C

2.1.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.1.8 Custody condition

Constant room at 4 °C for 7 days

2.1.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.1.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.1.11 Disposal of the resting substances

Scrap after test

2.1.12 Certificate of analysis

Client supplies to test organization

## **2.2 Test substance-2 (Non-treated ballast water (3-32 psu) -Control)**

2.2.1 Substance name

Non-treated water

2.2.2 Lot No.

none

2.2.3 Type

liquid

2.2.4 Principal ingredients

Seawater

2.2.5 Temperature

14~27 °C

2.2.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.2.8 Custody condition

Constant room at 4 °C for 7 days

2.2.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.2.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.2.11 Disposal of the resting substances

Scrap after test

2.2.12 Certificate of analysis

Client supplies to test organization

### **2.3 Test substance-3 (Treated ballast water (> 32 psu) -Treated)**

2.3.1 Substance name

Treated water

2.3.2 Lot No.

none

2.3.3 Type

liquid

2.3.4 Principal ingredients

Seawater

2.3.5 Temperature

14~27 °C

2.3.6 Salinity

> 32 psu

2.3.7 pH

6~8

2.3.8 Custody condition

Constant room at 4 °C for 7 days

2.3.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.3.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.3.11 Disposal of the resting substances

Scrap after test

2.3.12 Certificate of analysis

Client supplies to test organization

## **2.4 Test substance-4 (Non-treated ballast water (> 32 psu) -Control)**

2.4.1 Substance name

Non-treated water

2.4.2 Lot No.

none

2.4.3 Type

liquid

2.4.4 Principal ingredients

Seawater

2.4.5 Temperature

14~27 °C

2.4.6 Salinity

3-32 psu

2.3.7 pH

6~8

2.4.8 Custody condition

Constant room at 4 °C for 7 days

2.4.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.4.10 Responsible for sampling, handling and custody

Name: Marine Eco-technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.4.11 Disposal of the resting substances

Scrap after test

2.4.12 Certificate of analysis

Client supplies to test organization

## 2.5 Preparation of the test substance (SOP#TP-011)

The concentration of test substance is diluted on the control seawater (non-treated ballast water) as crude liquid. The six concentrations of test substance including 0.00 % (only non-treated ballast water) and 100.00 % are arranged for the final definitive experiment.

## 2.6 Culture condition

### 2.6.1 Hatching of test organisms (SOP#TO-007)

Test organisms	<i>Brachionus plicatilis</i>
Vessel	6 hole plate
Medium	20 psu filtered seawater (0.45µm membrane filter, Whatman)
Temperature	25 °C
Light source	“cool-white” fluorescent lamp
Light cycle	Continuous light
Light cycle	1,000 ~ 3,000 lux

The cysts were obtained from commercial supplier (See ‘2.7 Test system’).

### 2.6.2 Feeding regime

None

## 2.7 Test system

### 2.7.1 Test organisms

.1 Scientific name	<i>Brachionus plicatilis</i>
.2 Producer	MicroBioTests Inc., Belgium
.3 Dealer	Marine Eco-technology Institute Co., Ltd., Republic of Korea

### 2.7.2 Ground of selected test organisms

Marine rotifer, *Brachionus plicatilis* have the advantages of cosmopolitan distribution, wide salinity adaptability, important trophic roles as primary consumer in marine ecosystem, short generation time and easy culture in small spaces. For this reason *Brachionus plicatilis* is commonly used as a toxicity test.

### 2.7.3 Preparation of the test organisms (SOP#TO-007)

Rotifer cyst hatching is initiated approximately 24 h prior to the start of the test, and have to be carried out in seawater of reduced salinity (20 psu). They are incubated at 25 °C and 1,000 to 3,000 lux continuous illumination. They are checked the hatching hourly after 24 h incubation in order to collect the test animals



within 2 hours of their hatching. For the > 32 psu de-ballast water, rotifer neonates are acclimated for approximately 1 hour to adapt to the eventual salinity change.

#### 2.7.4 Check the test organisms for the test (SOP#TO-007)

Health of the test organisms such as the mortality, appearance and abnormal behavior is observed with a microscope (SZ51, Olympus).

#### 2.7.5 Disposal of the resting test organisms (SOP#TO-006)

The resting test organisms are scrapped after sterilization by the autoclave.

### 2.8 Study design

#### 2.8.1 Test concentration (SOP#TP-011)

Control	Control seawater	100.00 %: Seawater treated by BioViolet™	0.00 %
6.25 %	Control seawater	93.75 %: Seawater treated by BioViolet™	6.25 %
12.50 %	Control seawater	87.50 %: Seawater treated by BioViolet™	12.50 %
25.00 %	Control seawater	75.00 %: Seawater treated by BioViolet™	25.00 %
50.00 %	Control seawater	50.00 %: Seawater treated by BioViolet™	50.00 %
100.00 %	Control seawater	0.00 %: Seawater treated by BioViolet™	100.00 %

#### 2.8.2 Exposure conditions (SOP#TP-014)

*Brachionus plicatilis* is placed in test vessels containing food (*Chlorella vulgaris* ,  $1 \times 10^6$  cells/mL) divided into five neonates each replication of concentration including the control. And then the test vessels are placed in incubator for 96 hours at  $25 \pm 1^\circ\text{C}$ .

Experiment conditions and criteria are shown Annex 2 and application site is shown Annex 3.

#### 2.8.3 Observation item and measurement components

##### .1 Population growth rate (SOP#TP-014)

Each test vessel is checked for density of *Brachionus plicatilis* every 24 hours after the beginning of the experiment. Mortalities are recorded and then dead animals are removed.

Calculations of the population growth rate and the percentage inhibition growth are shown Annex 3.

##### .2 Other observations (SOP#TP-014)

In addition to immobility and mortality, any abnormal behavior and appearance are recorded.

##### .3 Temperature (SOP#TE-002)

Temperature of the solutions is measured at the beginning and end of the experiment.

#### .4 Dissolved oxygen (SOP#TE-002)

Dissolved oxygen (DO) of the solutions is measured at the beginning of the experiment.

(Test vessels contain only 1mL, it is technically difficult to measure DO at the end of the experiment. It is also known that brachionid rotifers are not sensitive to low oxygen levels.)

#### .5 pH (SOP#TE-002)

pH of the solutions is measured at the beginning and end of the experiment.

### **2.9 Statistical procedures (SOP#SP-101)**

LC50 values of the end point are estimated by statistical analysis procedure referred from USEPA (2002). TOXCALC 5.0 program (Tidepool scientific software, USA) is used for statistic analysis.

### **3. REFERENCES**

- ASTM. 2004. Standard guide for acute toxicity test with the rotifer *Brachionus*. E-1440-91. 8pp.
- U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.
- Janssen, C.R., G. Persoone and T.W. Snell. 1994. Cyst-based toxicity tests. VIII. Short-chronic toxicity tests with the freshwater rotifer *Brachionus calyciflorus*. Aquatic Toxicology, 28, 243-258.

## ANNEX 1

### Definitions

**1. Rotifer cyst** is that a rotifer embryo arrested at an early stage in development, enclosed in an envelope and resistant to desiccation and temperature extremes. Rotifer cysts are often incorrectly referred to as resting eggs. Upon hydration, embryonic development resumes until a neonate female emerges from the cyst.

**2. Rotifer neonate** is a newly hatched, freely swimming rotifer. All neonates hatched from cysts are females.

**3. EC<sub>x</sub>** is the concentration of the test substance dissolved in water that results in a x percent reduction in reproduction of *Brachionus plicatilis* within a stated exposure period.

**4. Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant effect on reproduction (at  $p < 0.05$ ) when compared with the control, within a stated exposure period.

**5. No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC.

## ANNEX 2

Experiment conditions and acceptability criteria for the rotifer, *Brachionus plicatilis* chronic population growth rate toxicity tests with the > 32 and 3-32 psu de-ballast water from the BioViolet™

Test parameter	Conditions
Test type	Static (non-renewal)
Temperature	25 ± 1 °C
Light quality	None
Light intensity	Darkness
Photoperiod	0h light: 24 h dark
Test chamber size	1.5 mL (48 hole plate)
Test solution volume	1 mL
Renewal of test solutions	None
Age of test organisms	Less than 2 hours
Number of individuals per test chamber	5 inds.
Replicate test chambers per concentration	12
Feeding regime	Once at the beginning of the experiment ( <i>Chlorella vulgaris</i> , 1 × 10 <sup>6</sup> cells/mL)
Cleaning	None
Aeration	None
Dilution water	Filtered seawater (Control seawater) by 0.45 µm membrane filter
Test concentration	5 including 100.00% effluents and a control
Dilution factor	Effluents: 0.5
Test duration	96 hours
Endpoints	population growth rate
Test acceptability criteria	- Population growth rate in the control : ≥ 0.55 - Percentage growth inhibition in the lowest toxicant concentration: < 50%

## ANNEX 3

**1. The population growth rate for a specific period is calculated from equation [1]:**

$$r_m = \frac{\ln N_t - \ln N_0}{t} \quad [1]$$

where:

$r_m$  is the intrinsic rate of natural increase in the population growth experiment.

$N_0$  and  $N_t$  are represented the population size at  $t=0$  and  $t=4$ , respectively.

**2. The percentage growth inhibition for each treatment replicate is calculated from equation [2]:**

$$I\% = \frac{N_{control} - N_{toxicant}}{N_{control}} \times 100 \quad [2]$$

where:

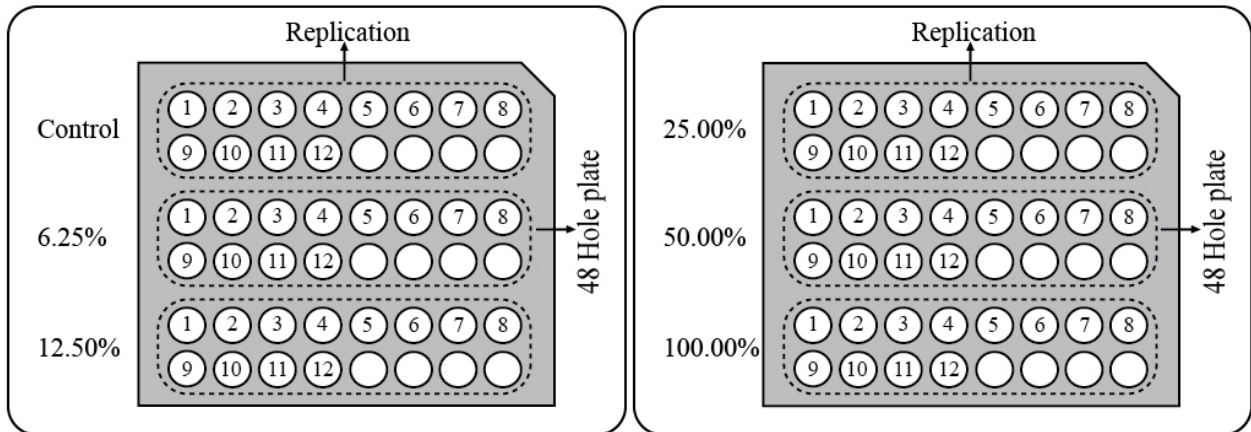
$I\%$  is percent inhibition in average growth rate.

$N$  are represented the population in each test concentrations.

## ANNEX 4

### Application Site

Application site for the chronic toxicity test with the rotifer, *Brachionus plicatilis* is shown as follow.



### **2.3.2 ANNEX 2**

**The olive flounder fish, *Paralichthys olivaceus*, Chronic Toxicity  
Test**



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485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea  
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Fax +82-51-611-0588

# STUDY PLAN

**Chronic Aquatic Toxicity For > 32 & 3-32 psu Treated Ballast Water of BioViolet™**

**- The olive flounder fish, *Paralichthys olivaceus*, Chronic Toxicity Test**

**Study Name: BioViolet™**

**Study No: BW- DBWT1107-KS**

Marine Eco-Technology Institute Co., Ltd.  
485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea



## STUDY PLAN PREPARATION AND APPROVAL

Study title: Chronic toxicity to assess the toxic effects of the > 32 & 3-32 psu of ballast water treated by the BioViolet™ toward survival of olive flounder fish, *Paralichthys olivaceus*

Study name: BioViolet™ Study No: BW-DBWT1107-KS

Test facility Marine Eco-technology Institute Co., Ltd.

2011. 02. 28.

Study Director: Jin Hee Kim

(signature)

Lab. Manager: Myung-Baek Shon

(signature)

Project Officer: Min Ho Son

(signature)

Organization Korean Marine Equipment Research Institute

2011. 02. 28.

Test Facility Manager: Jun-Hak Lee

(signature)

Project Director: Young-Soo Kim

(signature)

Kwang San Co., Ltd.

2011. 02. 28.

Monitor: Sang Bum Bae

(signature)

Sponsor: Young Woo Lee

(signature)

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# **1. OUTLINE OF STUDY**

## **1.1 Purpose**

The purpose of this study is to assess the chronic toxic effects of the > 32 and 3-32 psu ballast water treated by the BioViolet™ on the survival concerning early life stage of olive flounder, *Paralichthys olivaceus*.

## **1.2 Principle**

The embryo and sac-fry stages of olive flounder fish, *Paralichthys olivaceus* are exposed to the treated ballast water of the BioViolet™ for 7 days. The test is begun by placing fertilized eggs in the test chambers and terminated just before complete absorption of the yolk-sac of any larvae or mortalities by starvation start in any of the test chambers in the control. Lethal and sub-lethal effects are assessed and compared with the control.

## **1.3 Good Laboratory Practice**

All procedure of this study is complied with following GLP regulation:

- “OECD Principles of Good Laboratory Practice”

Organization for Economic Co-operation and Development, ENV/MC/CHEM(98)17 (as revised in 1997)

## **1.4 Guideline**

This study is complied with principle and conducted on the basis of following standard method and applied for olive flounder, *Paralichthys olivaceus*:

- “OECD Guideline for Testing of Chemicals, 212, Fish, Short-term Toxicity Test on the Embryo and Sac-fry Stages”

Organization for Economic Co-operation and Development, (Adopted: September. 21, 1998)

## **1.5 Sponsor**

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

Tel: +82-51-974-6351

Fax: +82-51-974-6405

## **1.6 Test facility**

Name: Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

Tel: + 82-51-611-6200

Fax: + 82-51-611-0588

## 1.7 Test site

Name: Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

## 1.8 Study director

Name: Jin Hee Kim

Department: 3<sup>rd</sup> research team

## 1.9 Study timetable

### 1.9.1 Treated ballast water (3-32 psu) and non-Treated ballast water (3-32 psu)

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 09. 27
Collect of sample	2011. 09. 26
Experimental start	2011. 09. 27
Exposure date	2011. 09. 27
Evaluation of mortality	2011. 09. 27 ~ 10. 04
Experimental completion	2011. 10. 04
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### 1.9.2 Treated ballast water (> 32 psu) and non-Treated ballast water (> 32 psu)

Study initiation	2011. 02. 28
Receipt of test organisms	2011. 11. 08
Collect of sample	2011. 11. 07
Experimental start	2011. 11. 08
Exposure date	2011. 11. 08
Evaluation of mortality	2011. 11. 08 ~ 11. 15
Experimental completion	2011. 11. 15
Draft of final report	2011. 12. 23
Final Report	2011. 12. 30
Study completion	2011. 12. 31

### **1.10 Responsible Personnel**

Laboratory manager (Test site manager)	Myung-Baek Shon
Statistical analysis	
Culture of test organisms	Tae Won Kim
Observation and record	
Sampling, transit and custody of test substance or solution	
Disposal of the resting substance	Je Kwan Park
Check the chemical condition	
Disposal of the resting organisms	Hyeong Ju Seok
Sampling, transit and custody of test substance or solution	

### **1.11 Retention of records and data**

#### **1.11.1 Duration**

Duration of storage is 5 years after end of the study. It will be decided that storage of data after 5 years should be consulted with the client.

#### **1.11.2 Place of storage**

Name: Archives of Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

#### **1.11.3 Retention of records and data**

Study plan, final report, raw data, documents related with the study, communication documents, and etc.

## 2. MATERIALS AND METHODS

### 2.1 Test substance-1 (Treated ballast water (3-32 psu) -Treated)

2.1.1 Substance name

Treated water

2.1.2 Lot No.

none

2.1.3 Type

liquid

2.1.4 Principal ingredients

Seawater

2.1.5 Temperature

14~27 °C

2.1.6 Salinity

3-32 psu

2.1.7 pH

6~8

2.1.8 Custody condition

Constant room at 4 °C for 7 days

2.1.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.1.10 Responsible for sampling, handling and custody

Name: Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.1.11 Disposal of the resting substances

Scrap after test

2.1.12 Certificate of analysis

Client supplies to test organization

## **2.2 Test substance-2 (Non-treated ballast water (3-32 psu) -Control)**

2.2.1 Substance name

Non-treated water

2.2.2 Lot No.

none

2.2.3 Type

liquid

2.2.4 Principal ingredients

Seawater

2.2.5 Temperature

14~27 °C

2.2.6 Salinity

3-32 psu

2.2.7 pH

6~8

2.2.8 Custody condition

Constant room at 4 °C for 7 days

2.2.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.2.10 Responsible for sampling, handling and custody

Name: Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.2.11 Disposal of the resting substances

Scrap after test

2.2.12 Certificate of analysis

Client supplies to test organization

### **2.3 Test substance-3 (Treated ballast water (> 32 psu) -Treated)**

2.3.1 Substance name

Treated water

2.3.2 Lot No.

none

2.3.3 Type

liquid

2.3.4 Principal ingredients

Seawater

2.3.5 Temperature

14~27 °C

2.3.6 Salinity

> 32 psu

2.3.7 pH

6~8

2.3.8 Custody condition

Constant room at 4 °C for 7 days

2.3.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.3.10 Responsible for sampling, handling and custody

Name: Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.3.11 Disposal of the resting substances

Scrap after test

2.3.12 Certificate of analysis

Client supplies to test organization



## **2.4 Test substance-4 (Non-treated ballast water (> 32 psu) -Control)**

2.4.1 Substance name

Non-treated water

2.4.2 Lot No.

none

2.4.3 Type

liquid

2.4.4 Principal ingredients

Seawater

2.4.5 Temperature

14~27 °C

2.4.6 Salinity

3-32 psu

2.4.7 pH

6~8

2.4.8 Custody condition

Constant room at 4 °C for 7 days

2.4.9 Supplier

Name: Kwang San Co., Ltd.

Address: 1173-2 Jisa-dong, Gangseo-gu, Pusan, Korea

2.4.10 Responsible for sampling, handling and custody

Name: Marine Eco-Technology Institute Co., Ltd.

Address: 485-1 Yongdang, Namgu, Busan, 608-830, Republic of Korea

2.4.11 Disposal of the resting substances

Scrap after test

2.4.12 Certificate of analysis

Client supplies to test organization

## 2.5 Preparation of the test substance (SOP#TP-011)

The concentration of test substance is diluted on the control seawater (non-treated ballast water) as crude liquid. The six concentrations of test substance including 0.00 % (only non-treated ballast water) and 100.00 % are arranged for the final definitive experiment.

## 2.6 Culture condition

### 2.6.1 Holding of test organisms

Test organisms	<i>Paralichthys olivaceus</i>
Culture type	-
Vessel	-
Medium	-
Temperature	-
Light source	-
Light cycle	-
Light intensity	-
Dissolved oxygen	-

The eggs were obtained from commercial supplier (See '2.7 Test system').

### 2.6.2 Feeding regime

None

## 2.7 Test system

### 2.7.1 Test organisms

.1 Scientific name	<i>Paralichthys olivaceus</i>
.2 Producer	KyeongYang Fisheries, Yo-Su city, Jeolla province, Republic of Korea
.3 Dealer	Marine Eco-Technology Institute Co., Ltd., Republic of Korea

### 2.7.2 Ground of selected test organisms

Pleuronectiformes Paralichthyidae *Paralichthys olivaceus* called olive flounder is widely distributed in coastal water of Korea and is important as economic marine products. *P. olivaceus* plays in marine trophic structure as a top predator.

### 2.7.3 Preparation of the test organisms (SOP#TP-007)

The fertilized eggs are obtained from commercial supplier. As the sensitivity of the experiment can be seriously influenced by delaying the start of the test, the experiment is initiated within 8 hours after

fertilization.

#### 2.7.4 Check the test organisms for the experiment (SOP#TP-007)

Health of the eggs such as the color and appearance is observed.

#### 2.7.5 Disposal of the remaining test organisms (SOP#TO-006)

The resting test organisms are scrapped after test.

## 2.8 Study design

### 2.8.1 Test concentration (SOP#TP-011)

Control	Control seawater	100.00 %: Seawater treated by BioViolet™	0.00 %
6.25 %	Control seawater	93.75 %: Seawater treated by BioViolet™	6.25 %
12.50 %	Control seawater	87.50 %: Seawater treated by BioViolet™	12.50 %
25.00 %	Control seawater	75.00 %: Seawater treated by BioViolet™	25.00 %
50.00 %	Control seawater	50.00 %: Seawater treated by BioViolet™	50.00 %
100.00 %	Control seawater	0.00 %: Seawater treated by BioViolet™	100.00 %

### 2.8.2 Exposure conditions (SOP#TP-007)

30 eggs are placed in the each replication of test vessel for the > 32 and 3-32 psu treated ballast water. And then the test vessels are placed in the constant room for 7 days at  $20 \pm 1^\circ\text{C}$ .

Experiment conditions and criteria are shown Annex 2 and application site is shown Annex 3.

### 2.8.3 Observation item and measurement components

#### .1 Hatching and survival (SOP#TP-007)

Eggs are considered dead if they are a marked loss of translucency and change in coloration. Embryos are considered dead if they are absence of body movement and/or absence of heart-beat and/or opaque discoloration. Larvae are considered dead if they have immobility and/or absence of respiration movement and/or absence of heart-beat and/or opaque coloration of central nervous system and/or lack of reaction mechanical stimulus. Dead eggs, embryos and larvae are removed after record.

#### .2 Length and weight (SOP#TP-010)

Total length and dry weights are measured at the end of the experiment.

#### .3 Stage of embryonic development (SOP#TP-007)

Embryonic development is observed and recorded as remark.

.4 Temperature (SOP#TE-002)

Temperature of the solutions on each vessel during the experiment period is measured daily.

.5 Dissolved oxygen (SOP#TE-002)

DO of the solutions on each vessel during the experiment period is measured daily.

.6 pH (SOP#TE-002)

pH of the solutions on each vessel during the experiment period is measured daily.

.7 Salinity (SOP#TE-002)

Salinity of the solutions during the experiment period is measured daily.

## **2.9 Statistical procedures (SOP#SP-101)**

NOEC, LOEC, LC25 and LC50 values of the end point are estimated by statistical analysis procedure referred from USEPA (2002). TOXCALC 5.0 program (Tidepool scientific software, USA) is used for statistic analysis.

## **3. REFERENCES**

OECD guidelines for the testing of chemicals-212. Fish, Short-term toxicity test on the embryo and sac-fry stages. 20pp.

U.S. EPA. 2002. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. 1200 Pennsylvania Avenue NW Washington, DC 20460.EPA-821-R-02-013.

## ANNEX 1

### Definitions

**1. Semi-static test** is a test without flow of solution, but with occasional batch wise renewal of the test solution after prolonged periods.

**2. LC25 and LC50** in this study is the median lethal concentration, i.e. that concentration of the test substance in water which kills 25 percent and 50 percent of a test batch of fish within a particular period of exposure.

**3. Lowest Observed Effect Concentration (LOEC)** is the lowest tested concentration at which the substance is observed to have a statistically significant reducing effect on survival (at  $p < 0.05$ ) when compared with the control, within a given exposure time. However, all test concentrations above the LOEC must have a harmful effect equal to or greater than those observed at the LOEC

**4. No Observed Effect Concentration (NOEC)** is the test concentration immediately below the LOEC.

## ANNEX 2

Experiment conditions and acceptability criteria for embryo and sac-fry stage of olive flounder, *Paralichthys olivaceus*, chronic mortality toxicity tests with the > 32 and 3-32 psu seawater treated by the BioViolet™

Test parameter	Conditions
Test type	Semi-static
Temperature	20±1 °C
Light quality	“Cool white” fluorescent lighting
Light intensity	2,000 lux
Photoperiod	16 h light: 8 h dark
Test chamber size	1 L
Test solution volume	0.8 L
Renewal of test solutions	Every other day
Age of test organisms	Less than 8 h after fertilization
Number of individuals per test chamber	30
Replicate test chambers per concentration	3
Feeding regime	None
Cleaning	Exchange the test water
Aeration	To maintain DO (> 60 % of the air saturation value)
Dilution water	Filtered seawater (Control seawater) by 1µm CP Filter, Chisso Filter
Test concentration	5 including 100.00% effluents and a control
Dilution factor	Effluents: 0.5
Test duration	7 days
Endpoints	Survival
Test acceptability criteria	<ul style="list-style-type: none"> <li>- The water temperature change: less than ± 1.5 °C</li> <li>- Overall survival of fertilized eggs in the controls: more than 70 %</li> </ul>

## ANNEX 3

### Application Site

Application site for the chronic toxicity test with the fertilized eggs of olive flounder, *Paralichthys olivaceus* is shown as follow.

